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THE HEMATOLOGIC CHANGES INDUCED IN GUINEA PIGS BY THE PROLONGED ADMINISTRATION OF PTEROYL GLUTAMIC ACID ANTAGONISTS

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ST. LOUIS, MO.

ANTAGONISTS of pteroyl glutamic acid (folic acid) are currently being evaluated as therapeutic agents for the treatment of leucemia and are being used experimentally in attempts to study the physiologic relationship between pteroyl glutamic acid (PGA) and the antipernicious anemia principle of liver. Detailed information about the hematologic effects produced by PGA antagonists is needed as a background for these observations. The experiments reported in this paper define the hematologic changes induced in guinea pigs by the prolonged administration of four different antagonists. An earlier report described the effects of relatively large doses of 4-amino pteroyl glutamic acid (4-amino PGA) and emphasized the striking difference in the susceptibility of different species of animals to the drug.[1] In the present study, small doses of the various antagonists were used in order that chronic rather than acute changes might be induced. Guinea pigs were selected because they occupy an intermediate position with respect to species susceptibility, and because the cytology of their peripheral blood and bone marrow is remarkably similar to that of the human being.

I. MATERIALS AND METHODS

Except where otherwise specified, adult guinea pigs weighing about 500 grams were used. Since the effects of PGA antagonists per se were being studied, no attempt was made to influence the amount of PGA available to the animals either through diet or intestinal synthesis. Lettuce and Purina Rabbit Chow were fed throughout the experiments. Antagonists were administered by subcutaneous injection at intervals of twenty-four hours.

Before injection of an antagonist was started, each animal was observed during a control period of two to five days to establish its normal range of weight and its peripheral blood values. Throughout the time of drug administration, frequent determinations were made of the red blood cell count, hemoglobin value, reticulocyte percentage, total and differential white blood cell counts, and the platelet level. For the reticulocyte and platelet estimations, Dameshek's platelet solution was used.[2] Hemoglobin was estimated by the Evelyn method.[3]

Bone marrow was aspirated from the iliac crest of guinea pigs in quantities sufficient to allow the preparation of satisfactory marrow films. A 20 gauge spinal puncture needle, complete with stilette, was cut to a length of one and one-half inches. The animals were firmly held, face down, with the hind legs fully extended; after shaving and cleansing the skin over the ilium, a small cut was made in the tough skin and the sterile needle introduced

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TABLE I. THE PERIPHERAL BLOOD OF NORMAL GUINEA PIGS

AUTHOR	NUMBER OF ESTIMATIONS	MEAN R.B.C. (MILLIONS/C. MM.) WITH RANGE	MEAN HB. WITH RANGE	MEAN RETICULOCYTE PERCENTAGE WITH RANGE	MEAN W.B.C./C. MM. WITH RANGE	MEAN PLATELETS (MILLIONS/C. MM.)	MEAN DIFFERENTIAL W.B.C. COUNT WITH RANGE				
							P. %	L. %	M. %	E. %	B. %
Scarborough[4]	529*	5.75 (4.5-6.8)	90-100 % (Sahli)	1-4			41.8	45.3	8.4		
	829*				10,700 (6,000-20,000)		41.8	45.3	8.4	4.8	0.77
Wintrobe[5]	4	5.75	14.5 Gm. %								
King and Lucas[6]	9 guinea pigs followed for 18-21 days	5.06 (4.7-5.25)			17,300 (13,000-23,000)		31.1 (0-76)	63.1 (20-97)	1.8 (0-10)	3.5 (0-14)	0.2 (0-2)
Present investigation	48	5.63 (4.4-6.7)	15.2 Gm. % (13.6-17.9)	1.2 (0.1-3.0)	10,600 (5,000-18,700)	1.7	44.7 (20-76)	47.6 (21-74)	5.2 (1-12)	1.2 (0-4)	1.3 (0-5)

*Figures collected from the literature.

through the muscle until contact was made with the crest of the bone. A boring movement allowed the tip of the needle to enter the marrow cavity, so that on withdrawal of the stilette, a small quantity of marrow blood and flecks could be aspirated into a 5 c.c. syringe. The flecks were spread on slides and stained with Wright's stain. In about 75 per cent of aspirations attempted in this manner, enough marrow was obtained for satisfactory examination. The operation appeared to cause little lasting discomfort to the animals, and although it was not felt desirable to repeat the procedure on the same side until at least ten days had elapsed, aspiration from the opposite ilium was carried out on the next day in some animals where rapid changes in the marrow cytology were anticipated.

No guinea pigs were sacrificed during the investigation, though several died from the effects of the drugs. Autopsy was then performed as soon as possible and the weight and gross appearances of the organs were noted. Fixed sections of liver, spleen, kidney, suprarenal, heart, and lung were made for microscopic examination.

(a) The Peripheral Blood and Bone Marrow of Normal Guinea Pigs.—Normal values for the cellular elements in the peripheral blood of guinea pigs are summarized in Table I. The tabulation includes data obtained by other workers as well as those from the present investigation.

The marrow in healthy guinea pigs is cytologically similar to normal human marrow (Fig. 1). It has been described in detail by Epstein and Tompkins[7]; figures given by them for the differential counts agree essentially with those found for the normal animals in the present study. Instead of reporting results in terms of differential counts, however, it was decided to describe the alterations induced by the PGA antagonists. This was done for two reasons: (1) careful descriptions of the appearance and distribution of the cell types probably convey a more accurate impression of any cytologic changes, and (2) in the treated animals, primitive cells were often encountered which were difficult to classify with conviction.

*(b) The Pteroyl Glutamic Acid Antagonists Used**.—The chemical names of the four antagonists employed in this investigation and the symbols which will be used to identify them are tabulated below:

NAME	SYMBOL
Pteroyl aspartic acid—racemic form	An-Fol-R†
Pteroyl aspartic acid—active form	An-Fol-A†
7 methyl pteroic acid	Met-Fol-B†
4-amino pteroyl glutamic acid	Aminopterin†; 4-amino PGA

Each of the first three antagonists was given alone to some of the animals and in combination with 4-amino PGA to others.

II. RESULTS

A. Pteroyl Aspartic Acid—R.—This compound was given to four guinea pigs. One animal (Guinea pig 1) received a daily dose of 20 mg., another (Guinea pig 3), 10 mg., and the other two (Guinea pigs 2 and 4), 5 mg. each. These dosages were continued for seventy-four, seventy-two, sixty-two, and fifty-three days respectively. No general ill effects were noted throughout the experiment; the animals maintained their weight, appetite, and normal activity.

In all four animals a mild, prolonged reticulocytosis, ranging from 4 to 13 per cent, developed after about ten days and continued for twenty to thirty days. In three animals (Guinea pigs 1, 3, and 4) this reticulocytosis was pre-

*Supplied by the Lederle Laboratories Division of the American Cyanamid Company, to whom the authors express their appreciation.

†Names used by the Lederle Laboratories to identify these compounds.

ceded by a fall in the erythrocytes to approximately 4 million per cubic millimeter, followed by a spontaneous return to normal levels. Otherwise, no changes

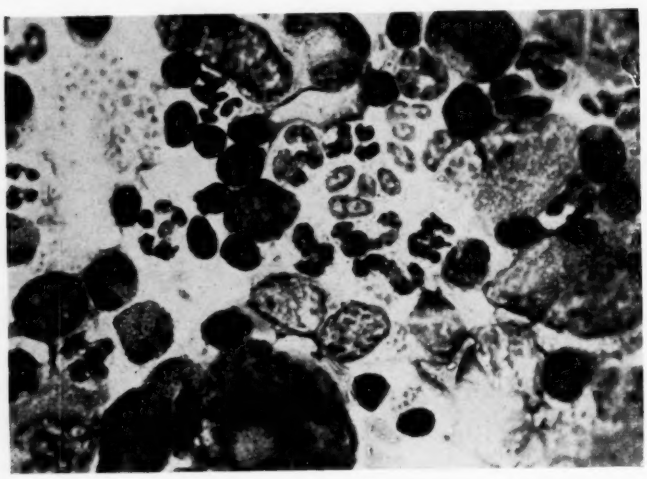


Fig. 1.—Marrow of a normal guinea pig (×850).

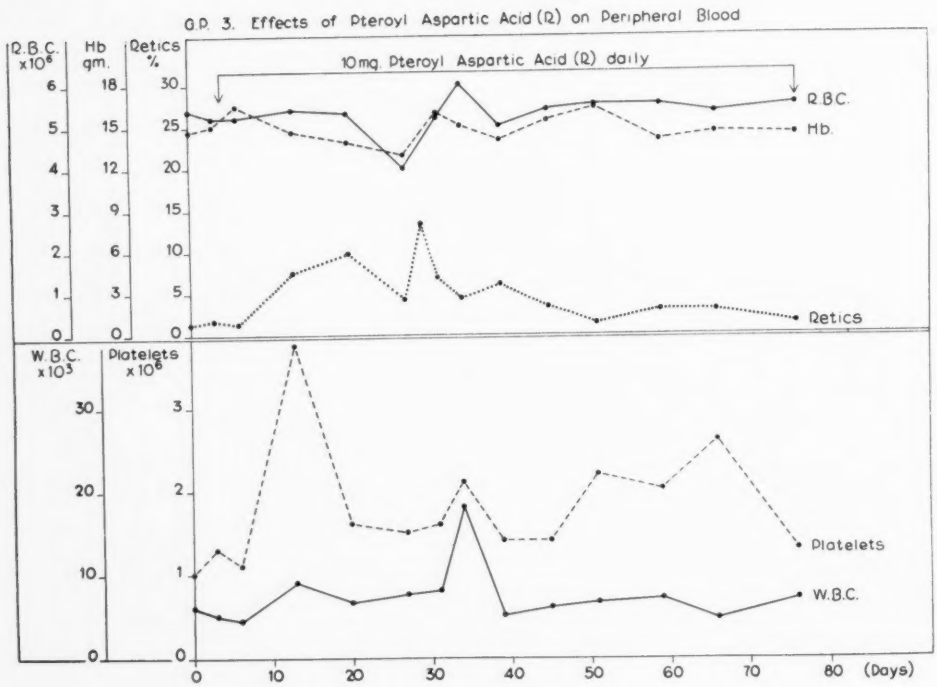


Fig. 2.

were noted in the peripheral blood of any of the animals; the white blood cell, differential and platelet counts remained within normal limits throughout. Hematologic data for Guinea pig 3 are graphically recorded in Fig. 2.

Bone marrow aspiration was successful in three of these animals (Guinea pigs 1, 2, and 4) at the completion of the experiment. Apart from a slight increase in the orthochromatic and polychromatic normoblasts, no morphologic change was observed.

B. Pteroyl Aspartic Acid—A.—This optically active form of pteroyl aspartic acid was administered to one animal (Guinea pig 5) in daily dosage of 20 mg. for sixty-seven days. No general ill effects were observed and the only hematologic change observed in the peripheral blood was an intermittent polymorphonuclear leucocytosis rising to levels as high as 28,000 cells per cubic millimeter. Marrow aspiration at the end of the experiment showed no abnormality other than an increase in metamyelocytes, band forms, and polymorphonuclear neutrophiles.

C. 7-Methyl Pteroic Acid.—Two animals (Guinea pigs 6 and 7) were given daily doses of 5 mg. and 10 mg. of this drug, respectively. After forty days the dosage was increased to 10 mg. and 20 mg. daily and continued for an additional twenty -two days. No general ill effects were noted.

On the lower dosage both pigs showed a mild reticulocytosis, ranging from 5 to 8 per cent, which appeared after seven days and continued for approximately twenty-five days. The red corpuscles remained within normal limits during the experiment. The total leucocyte count in both animals rose within ten days from control levels of 10,000 to 15,000 per cubic millimeter to around 20,000 per cubic millimeter and remained thus while the drug was being given. This slight increase affected both the granular and nongranular forms. The platelet values showed no striking change.

Marrow aspiration was unsuccessful in these animals.

D. 4-Amino Pteroyl Glutamic Acid.—4-amino PGA was given to eight animals (Guinea pigs 8 to 15), in six of which (Guinea pigs 8 to 13) the effects of prolonged daily administration were observed.

Three animals (Guinea pigs 8 to 10) received 0.25 mg. daily for ninety-two, sixty-five, and sixty-three days respectively. Guinea pigs 9 and 10 maintained their weight and appetite throughout. In Guinea pig 9 the red blood count had fallen gradually to 3.5 million per cubic millimeter after sixty-five days, but otherwise neither of these two animals showed any hematologic changes. Guinea pig 8 maintained its weight for sixty-four days on the 0.25 mg. dose, but thereafter lost weight steadily and died on the ninety-second day. During this whole period the red blood cells remained essentially normal. About the fiftieth day a leucocytosis of 23,000 cells per cubic millimeter developed because of an increase in both neutrophiles and lymphocytes. The elevated white blood cell count continued until the ninetieth day, though after the eightieth day it was accounted for entirely by a granulocyte increase, the lymphocytes falling sharply at this time. In the terminal two days, a dramatic fall occurred in the total white blood cells (from 24,250 to 2,350 cells per cubic millimeter) and in the platelets (Fig. 3).

Marrow studies were made in the latter animal eight days before death and post mortem. At the time of the peripheral leucocytosis, the marrow showed a marked increase in metamyelocytes, band and segmented neutrophiles (Fig.

4,*A*); although the red blood count at this time was normal, there was a definite increase in pronormoblasts and basophilic normoblasts. Megakaryocytes of normal appearance were seen. There was also present a number of large cells, of irregular outline with nongranular, pale blue-staining cytoplasm and large nuclei with an open chromatin network and occasional nucleoli. For reasons to be discussed later, these cells were considered to be "reticulum cells."

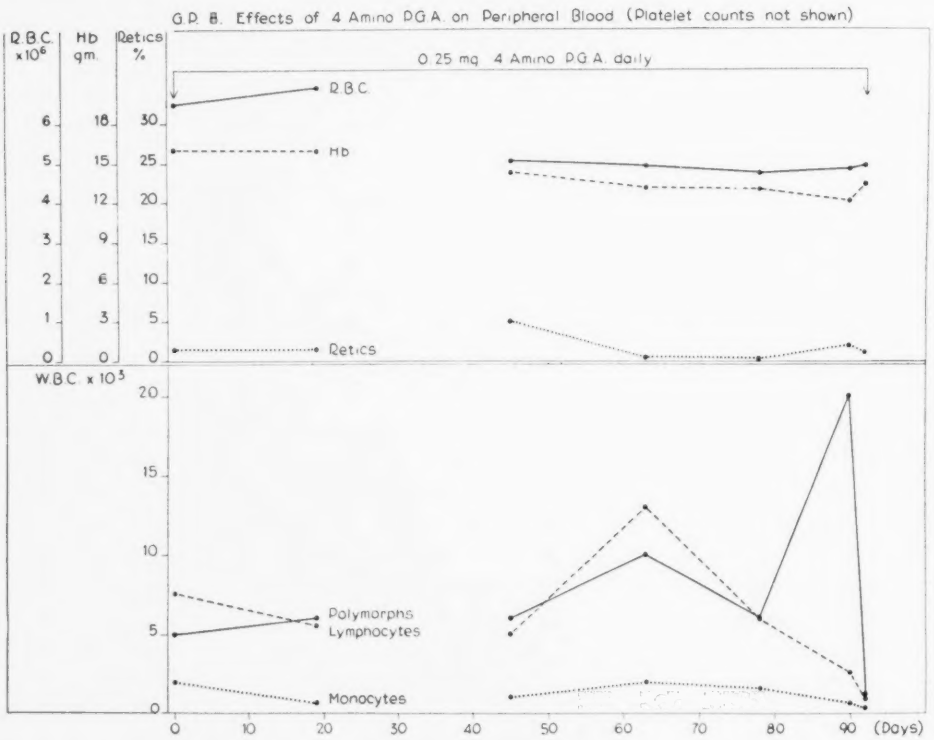


Fig. 3.

In the post-mortem marrow of the same animal (Guinea pig 8, Fig. 4,*B*), myelocytes were still present in considerable numbers but there was an almost total absence of metamyelocytes and polymorphonuclear granulocytes. The number of reticulum cells had definitely increased; their wide distribution and the general cellularity of the marrow could best be appreciated in fixed section. (Fig. 4,*C*.)

No abnormalities of the viscera were noted grossly at autopsy and no hemorrhages were observed macroscopically. On microscopic examination the liver showed extreme fatty change affecting the central and mid-zonal cells of the lobules. (Fig. 4,*D*.) An area of recent hemorrhage was seen in the medulla of one suprarenal. In the splenic pulp the endothelial elements were prominent, but apart from these findings the organs showed no changes.

Three animals (Guinea pigs 11, 12, and 13) were given prolonged daily dosage of 0.5 mg. 4-amino PGA.

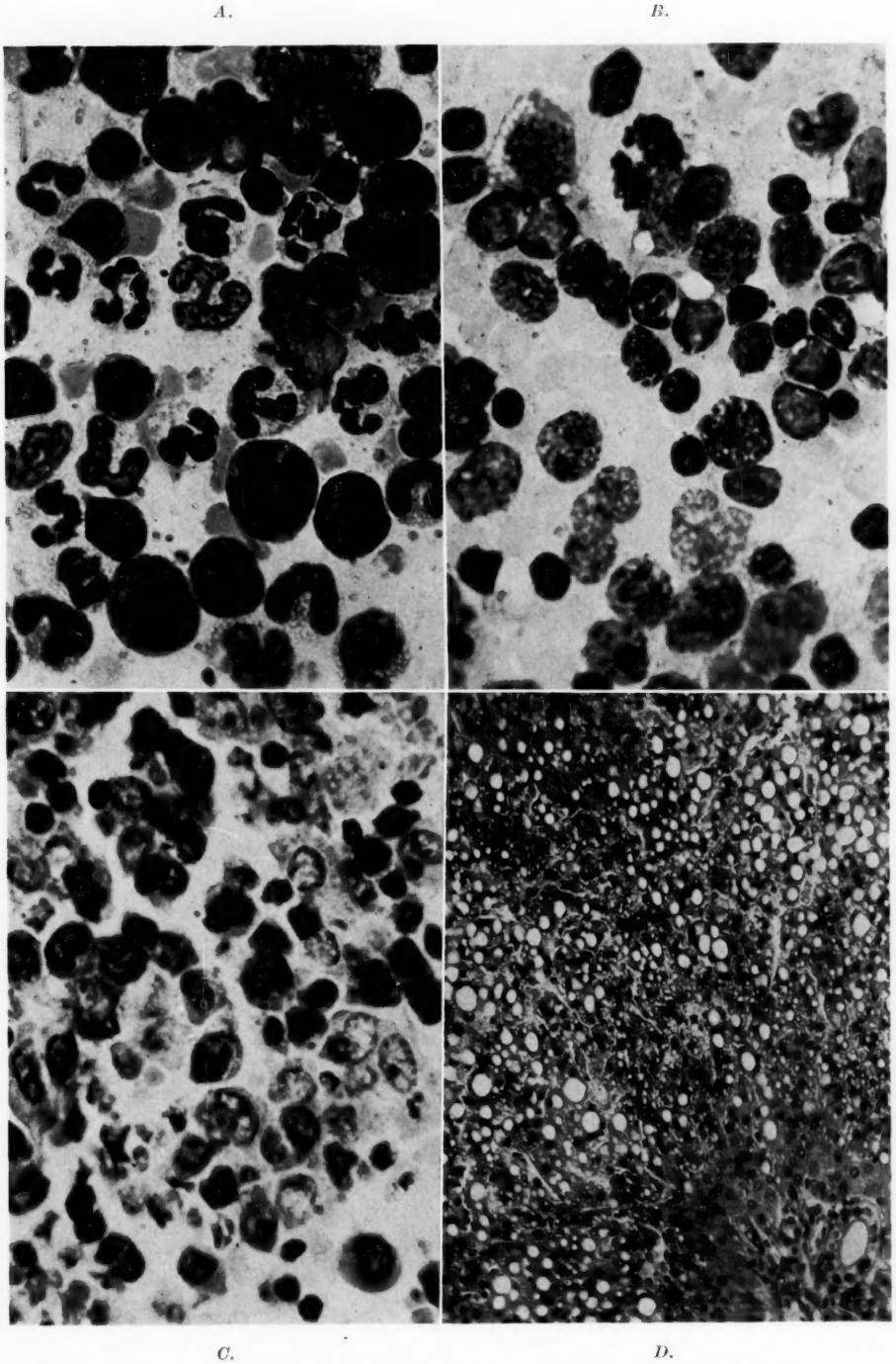


Fig. 4.—Guinea pig 8. *A*, Bone marrow during peripheral leucocytosis (×850). *B*, Bone marrow film after terminal leucopenia (post mortem, ×850). *C*, Bone marrow section after terminal leucopenia (post mortem, ×850). *D*, Liver (×170).

The first of these lost weight and died in thirty-two days. During this time its red blood cell count fell to 3.7 million per cubic millimeter and its leucocytes to 3,250 per cubic millimeter; the differential count showed a reduction in both granulocytes and lymphocytes. There was also a terminal drop in platelets. At the time of death, the marrow was very cellular and contained a large number of reticulum cells. The red cell precursors were decreased, and in the granulocytic series there was an almost complete absence of segmented and band forms. Myelocytes, however, were numerous, with basophile and eosinophile forms especially prominent. Megakaryocytes were scanty. Microscopic study of tissue sections showed areas of recent hemorrhage and edema in the lungs and an increase in the endothelial cells in the spleen. No fatty change was observed in the liver, and other organs examined showed no significant abnormalities.

The second animal (Guinea pig 12) was given 0.5 mg. 4-amino PGA for forty-eight days, at the end of which time it appeared to have suffered no general ill effects. No changes were observed in the peripheral blood other than a very gradual decline in the erythrocyte level to 4.22 million per cubic millimeter.

The third animal (Guinea pig 13 received the daily 0.5 mg. dosage for 144 days with no apparent change in its general condition. In the peripheral blood, a fall in erythrocytes to 3.03 million per cubic millimeter in thirty-six days was partly corrected by a subsequent reticulocytosis which continued for thirty days and attained a maximum of 15 per cent. Thereafter, the red blood cell level remained between 3.5 million and 4.0 million per cubic millimeter until the one hundred sixteenth day. While administration of 4-amino PGA was continued, 10 mg. PGA were then injected daily for fourteen days, followed by 5 U.S.P. units of liver extract daily for an additional two weeks. These supplements caused no increase in reticulocytes, and the red blood cell count fell gradually to 2.9 million per cubic millimeter. Up to this time (one hundred forty-fourth day) no change had occurred in the total or differential leucocyte count or in the number of platelets. Marrow aspiration studies were made on the one hundred sixteenth day and one hundred forty-fourth day, i.e. before and after the PGA and liver extract. On the one hundred sixteenth day the marrow was very cellular and showed erythroid hyperplasia with a large number of pronormoblasts and basophilic and polychromatic normoblasts. The granulocytic precursors and megakaryocytes appeared normal. A considerable number of reticulum cells was present. On the one hundred forty-fourth day, the marrow cytology was essentially unchanged (Fig. 5, *A* and *B*).

In two animals (Guinea pigs 14 and 15) the effects of giving a large single dose of 4-amino PGA were investigated.

Guinea pig 14, weighing 510 grams, received an injection of 20 mg. of the compound. No changes were observed in its general condition or in its peripheral blood during the succeeding fourteen days; aspirated marrow examined on the tenth day following the injection showed no abnormality. A second injection of 20 mg. produced the same negative result. Doses of 2 mg. were then administered daily for ten days; the white blood cell count fell to 5,500 per cubic millimeter but there was little change in the red blood cell and hemoglobin levels. The dosage was next reduced to 1 mg. daily for thirty-two days.

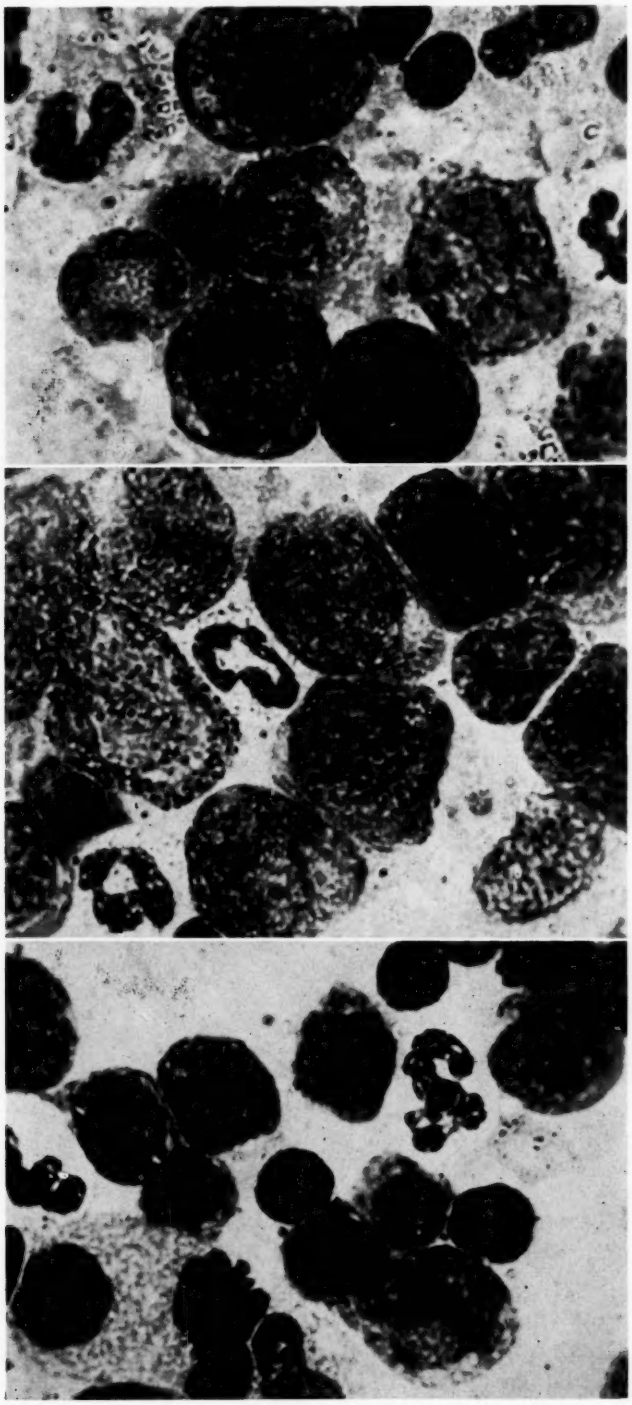
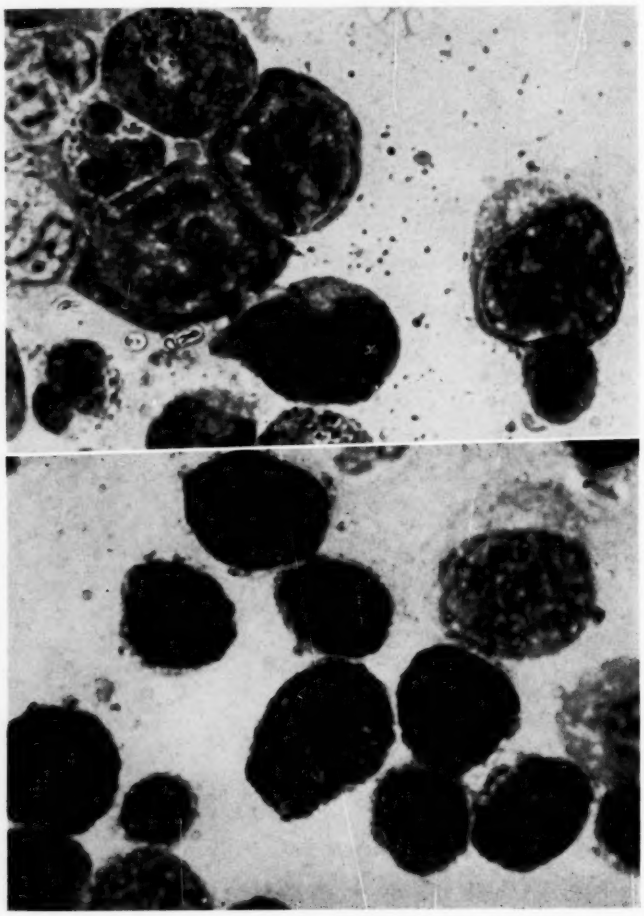


Fig. 5.—Guinea pig 13. *A*, Bone marrow after 0.5 mg. 4-amino PGA had been given daily for 116 days (×1400). *B*, Bone marrow after PGA and liver extract one hundred forty-fourth day) (×1400). *C*, Bone marrow five days after stopping 4-amino PGA (×1400).

During this time, the red blood cells decreased to 2.68 million per cubic millimeter and the hemoglobin level fell to 7.8 Gm. per cent; no further depression occurred in the leucocytes, and the differential and platelet counts remained normal. Marrow examination at the end of the period showed an increase in

A.



B.

Fig. 6.—Guinea pig 14. *A*, Bone marrow before stopping 4-amino PGA (×1400). *B*, Bone marrow twenty-four hours after stopping 4-amino PGA (×1400).

the number of pronormoblasts and basophilic and polychromatic normoblasts, with no change in the white cell precursors or megakaryocytes. Reticulum cells and plasma cells were prominent (Fig. 6,*A*).

The second animal (Guinea pig 15, 830 grams) was given a single injection of 40 mg. of 4-amino PGA. Observations over the next fourteen days revealed no general or hematologic effects. Doses of 4 mg. daily were then given for ten days, and while no changes were found in the peripheral blood, the marrow at the end of this time showed a decrease in the proportion of erythroid precursors with pronormoblasts and basophilic normoblasts outnumbering the later forms.

In the granulocytic series, all stages of precursors were present, though the proportion of early myelocytes was increased. Reticulum cells, plasma cells, and lymphocytes were numerous.

E. 4-Amino PGA and Pteroyl Aspartic Acid—R.—The combined effects of these two antagonists were studied in five animals (Guinea pigs 16 to 20). Table II indicates the daily dosage of each drug, the duration of administration,

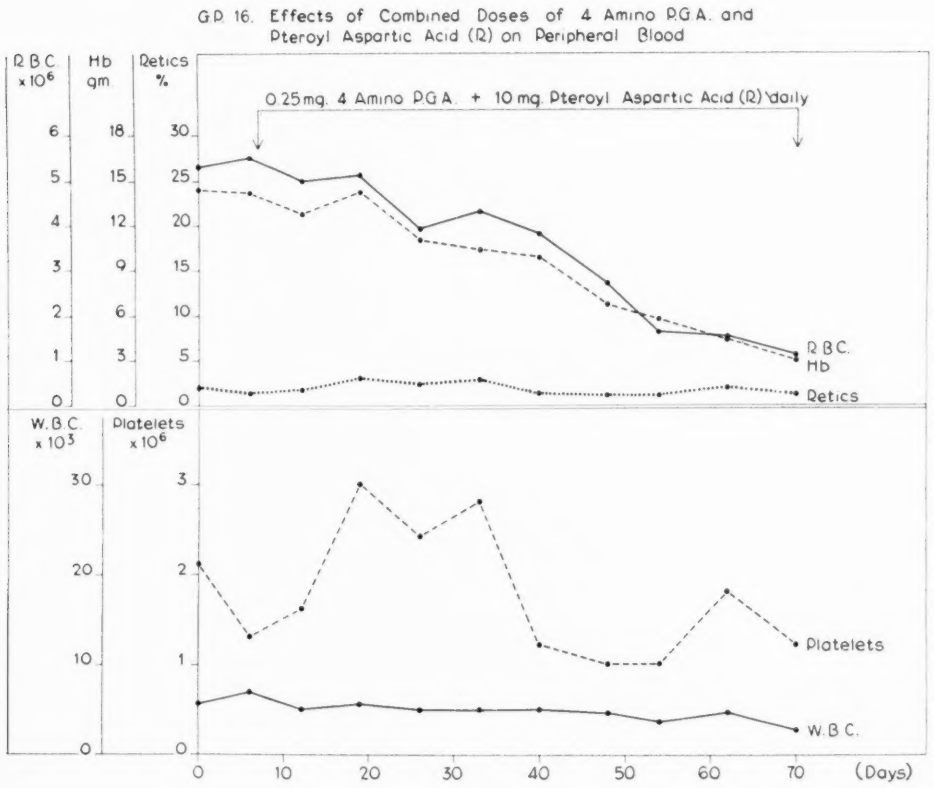


Fig. 7.

and the findings in the peripheral blood before and after the experiments. In all cases a general depression of erythrocytes, leucocytes, and platelets resulted. A severe anemia developed in animals 16 and 19 and marked leucopenia in Guinea pigs 16 and 18. In both of the latter, differential counts showed an almost equal reduction in granulocytes and lymphocytes. Weight loss occurred in Guinea pigs 16 and 18, though animal 16 was the only animal in the series to die (Fig. 7).

Marrow examined at the end of dosage in Guinea pigs 16, 17, 19, and 20 was cellular in each instance. There was an increase in pronormoblasts and basophilic and polychromatic normoblasts, with a diminution in orthochromatic normoblasts. In the anemic Guinea pig 16, the total erythroid elements were reduced with pronormoblasts as the predominating type. The granulocytic

TABLE II. EFFECTS ON THE PERIPHERAL BLOOD OF COMBINED DOSAGE WITH AMINOPTERIN AND AN-FOL-R

GUINEA PIG	DAILY DOSE OF AMINOPTERIN (MG.)	DAILY DOSE OF AN-FOL-R (MG.)	DURATION OF DOSAGE (DAYS)	R.B.C. (MILLIONS/C. MM.)		HB. (GM. %)		W.B.C./C. MM.		PLATELET (MILLIONS/C. MM.)	
				BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER
16	0.25	10	64	5.47	1.09	14.2	3.1	6,700	2,450	1.35	1.17
17	0.25	10	57	5.34	4.23*	14.0	11.1	7,500	4,950	2.35	1.75
18	0.25	5	48	6.43	4.73	14.8	12.2	6,800	1,900	2.60	0.52
19	0.25	20	25	4.68	4.69	14.9	13.4	10,100	11,400	3.25	2.60
	0.5	20	38	4.69	2.24	13.4	6.4	11,600	8,300	2.40	1.30
20	0.25	5	109	5.67	4.31†	15.1	11.7	16,500	12,300	3.0	1.30
	0.25	10	25	4.31	3.55	11.7	10.1	12,600	10,500	1.30	1.10

*R.B.C. fell to 3.31 millions/c.mm. but rose again to 4.23 millions/cmm. following spontaneous reticulocytosis reaching 20.5 per cent.
†R.B.C. fell to 3.62 millions/c.mm. but rose again to 4.31 millions/cmm. following spontaneous reticulocytosis reaching 20.6 per cent.

TABLE III. EFFECTS ON THE PERIPHERAL BLOOD OF COMBINED DOSAGE WITH AMINOPTERIN AND MET-FOL-B

GUINEA PIG	DAILY DOSE OF AMINOPTERIN (MG.)	DAILY DOSE OF MET-FOL-B (MG.)	DURATION OF DOSAGE (DAYS)	R.B.C. (MILLIONS/C. MM.)		HB. (GM. %)		W.B.C./C. MM.		PLATELETS (MILLIONS/C. MM.)	
				BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER
23	0.25	10	42	5.27	4.13	15.4	11.4	15,300	200	1.8	0.14
24	0.25	20	65	5.63	1.93	16.3	6.4	19,700	3,950	2.0	0.4
25	0.25	20	20	6.08	3.97	16.7	10.4	12,100	1,400	1.7	0.1

precursors appeared normal except for a marked decrease in polymorphonuclear leucocytes and band forms in Guinea pig 16. All four marrows showed megakaryocytes of normal appearance. Reticulum cells were numerous in every instance.

Post-mortem examination in animal 16 showed a large, pale liver weighing 24 grams and splenic enlargement to about three times the normal size. There were no signs of hemorrhage. Microscopically there was a marked degree of fatty change in the liver, with vacuolation of the central and mid-zonal cells of the lobules. Endothelial elements in the spleen were prominent. Other organs showed no significant changes.

F. 4-Amino PGA and Pteroyl Aspartic Acid—Active Form.—Two animals (Guinea pigs 21 and 22) were given daily injections of 0.25 mg. of 4-amino PGA and 20 mg. An-Fol-A.

Both lost weight; Guinea pig 21 died after twenty-four days and Guinea pig 22, at the end of forty-six days. Peripheral blood examinations showed a gradual decline in erythrocytes and in hemoglobin though at the time of death neither animal was severely anemic (3.67 million and 3.39 million per cubic millimeter). The white blood cell count for Guinea pig 21 fell from an initial value of 18,000 per cubic millimeter to 7,500 two days before death; the absolute number of lymphocytes decreased from 15,000 to 600 per cubic millimeter. Unfortunately, no terminal white blood count was obtained. Guinea pig 22 developed a leucopenia (1,450 per cubic millimeter) before death, the differential count showing a parallel fall in neutrophiles and lymphocytes. Platelets remained virtually unchanged.

Marrow examination at the time of death showed in both guinea pigs a decided increase in the number of pronormoblasts and early myelocytes. The more mature granulocytic precursors were considerably reduced and megakaryocytes were present though scanty. Reticulum cells were numerous; plasma cells and lymphocytes were found in more than normal numbers.

Necropsy revealed no macroscopic signs of hemorrhage in either animal. The liver was large in Guinea pig 21 (weight, 23 grams), but showed no histologic abnormality. There was increased endothelial activity in the splenic pulp of Guinea pig 22. No other abnormalities were observed in either animal.

G. 4-Amino PGA and 7 Methyl Pteroic Acid.—These two antagonists were administered simultaneously to three animals (Guinea pigs 23, 24, and 25). The amounts, duration, and effects on the peripheral blood are shown in Table III.

All three animals died, Guinea pigs 23 and 25 showing marked loss of weight. Leucopenia and thrombocytopenia developed terminally and there was some depression of erythrocytes in each pig; the anemia in Guinea pig 24 was severe. Changes in the leucocytes followed a general pattern: first there was an increase in the neutrophiles and monocytes with no change in the lymphocytes, then a sharp fall occurred in all three white cell types (Fig. 8).

In Guinea pigs 23 and 25 the bone marrow became uniformly hypoplastic for erythrocytic, granulocytic, and megakaryocytic elements; the most prominent cells were reticulum cells, plasma cells, and lymphocytes. Of the erythroid and

myeloid series, the majority were early forms: pronormoblasts and young myelocytes. The marrow of the severely anemic animal (Guinea pig 24) was actively cellular and showed an increase in the pronormoblasts and basophilic normoblasts with relatively few more mature forms. Considerable numbers of very early myelocytes were seen together with bands and segmented neutrophiles,

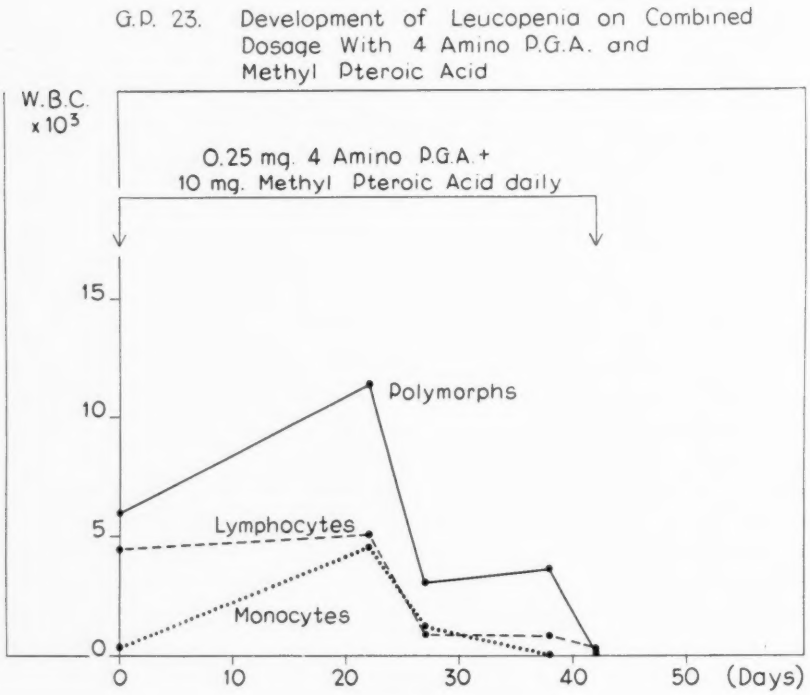


Fig. 8.

but the intermediate myelocyte and metamyelocyte stages were largely missing. Megakaryocytes were of normal appearance. In this marrow also, reticulum and plasma cells were numerous.

Macroscopic and microscopic examination of the tissues of these three animals showed little of note except for endothelial proliferation in the splenic pulp and a moderate degree of fatty change toward the center of the liver lobules in Guinea pigs 24 and 25.

H. Hematologic Recovery From Effects of Antagonists.—The peripheral blood and marrow changes which occurred when administration of the antagonist compounds was discontinued were observed in six of the animals: in Guinea pigs 13 and 14 after 4-amino PGA alone, and in Guinea pigs 17, 18, 19, and 20 after combined dosage with 4-amino PGA and An-Fol-R.

Hematologic recovery occurred with striking rapidity in each instance (Table IV). Figs. 9 and 10 show the detailed course of recovery from anemia and leucopenia in Guinea pigs 14 and 18 respectively. In every animal a reticulocytosis was evident within forty-eight hours after the last injection and reached a maximum (amounting to over 40 per cent in Guinea pigs 14 and 19)

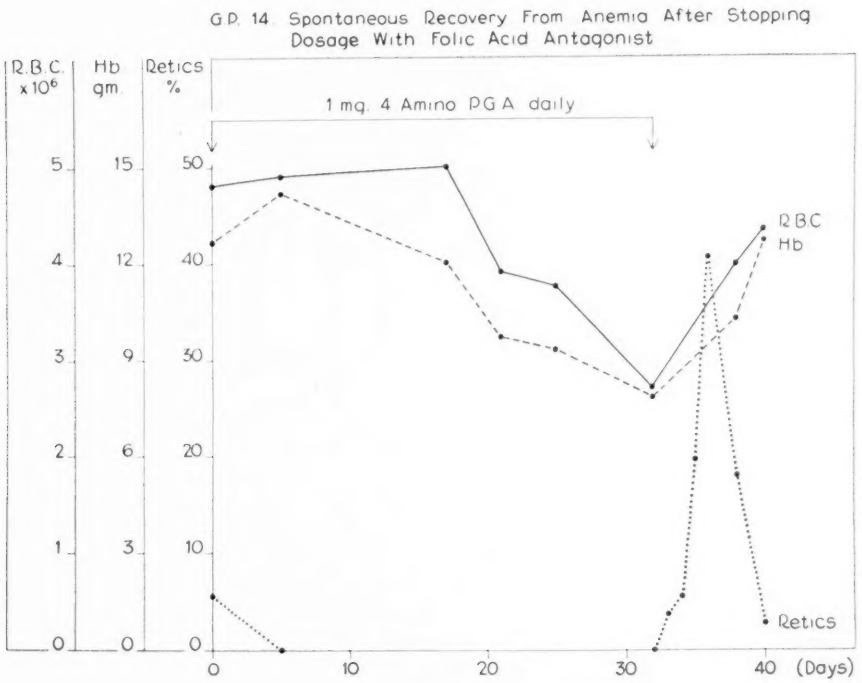

G.P. 14 Spontaneous Recovery From Anemia After Stopping Dosage With Folic Acid Antagonist
R.B.C. x10⁶
Hb gm.
Retics %
1 mg. 4 Amino P.G.A. daily
R.B.C.
Hb
Retics
(Days)

Fig. 9.

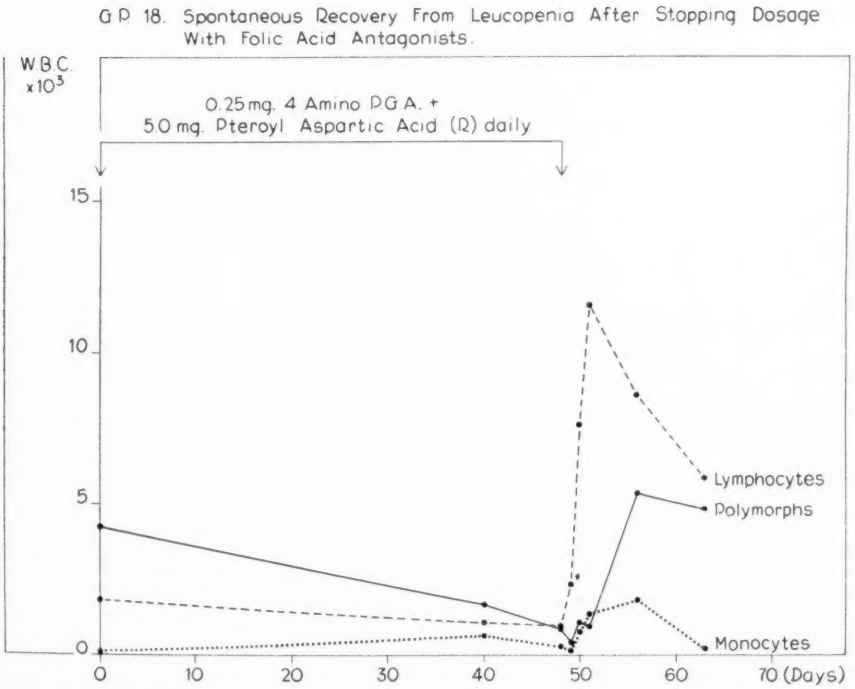

G.P. 18. Spontaneous Recovery From Leucopenia After Stopping Dosage With Folic Acid Antagonists.
W.B.C. x10³
0.25 mg. 4 Amino P.G.A. +
5.0 mg. Pteroyl Aspartic Acid (R) daily
Lymphocytes
Polymorphs
Monocytes
(Days)

Fig. 10.

TABLE IV. PERIPHERAL BLOOD FINDINGS BEFORE AND AFTER STOPPING DOSAGE OF FOLIC ACID ANTAGONISTS (FIGURES IN PARENTHESIS INDICATE NUMBER OF DAYS FOLLOWING CESSATION OF DRUGS)

GUINEA PIG	R.B.C. (MILLIONS/C. MM.)		HB. (GM. %)		RETICULOCYTE PERCENTAGE		W.B.C./C. MM.		PLATELETS (MILLIONS/C. MM.)	
	BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER
13	2.9	6.33 (12)	10.7	14.6 (12)	0.2	12.4 (3)	8,600	18,400 (19)	1.45	1.95 (12)
14	2.68	4.35 (8)	7.8	12.7 (8)	0.0	40.8 (4)	8,700	9,650	1.0	0.75 (8)
17	4.23	4.72 (10)	11.1	13.4 (10)	1.0	15.5 (3)	4,950	11,550 (10)	1.75	3.25 (3)
18	4.73	5.87 (8)	12.2	15.2 (15)	1.7	6.2 (4)	1,900	16,250 (8)	0.52	1.5 (8)
19	2.24	5.10 (6)	6.4	11.8 (10)	0.1	43.1 (5)	8,300	16,700 (3)	1.30	1.85 (3)
20	3.55	4.83 (10)	10.1	13.0 (10)	0.1	14.1 (3)	10,450	18,300 (10)	1.10	2.40 (10)

within three to five days. There was a rapid return of the red blood cell count toward normal, with a noticeable lag in the rate of hemoglobin recovery in some instances. In all the animals, cessation of the drug was followed also by a rise in leucocytes. Lymphocytes were the first cells to increase in number in every instance (Fig. 10).

Marrow was studied in three animals at different stages of the recovery phase. In Guinea pig 14, marrow was obtained twenty-four hours after the last dose of 4-amino PGA. Compared with the marrow of the previous day, there were definite signs of increased maturity of the erythrocytic precursors, with polychromatic and orthochromatic normoblasts more numerous although large numbers of pronormoblasts still remained (Fig. 5,*B* and *C*). A complete return to normal erythropoiesis was evident in the marrow of Guinea pig 19 obtained ten days after the last administration of 4-amino PGA and An-Fol-R.

III. DISCUSSION

The purpose of this discussion is to examine the experimental results obtained and to determine whether they indicate the mode of action of the PGA antagonists.

4-Amino PGA.—Of the four compounds employed in this investigation, 4-amino PGA was obviously the most active hematologically. The smallest dose (0.25 mg. daily) produced recognizable effects only when given over a very long period (nine to thirteen weeks), but when 0.5 mg. was administered daily, marrow and blood changes appeared much earlier.

In the bone marrow the effects of 4-amino PGA were characterized by an increasing number of early erythrocytic and/or granulocytic precursors. The general cellularity tended to be increased rather than decreased. In all instances the change toward immaturity was accompanied by the appearance of a great many large, primitive-looking cells with pale, nongranular cytoplasm and large nuclei with an open chromatin meshwork. The cytoplasm of these cells was sometimes vacuolated; their nuclei usually showed nucleoli and were not infrequently divided. These cells were seen only occasionally in normal guinea

pig marrow. It was decided that they were most probably reticulum cells since their morphologic characteristics corresponded with the description of the latter given by Jaffé[8] and other workers. The earlier red blood cell precursors were studied with a view to ascertaining whether dosage with a PGA antagonist would produce a maturation arrest of the type seen in pernicious anemia. Whereas large numbers of pronormoblasts were found, no cells were seen which possessed all of the cytologic features of the megaloblasts of pernicious anemia.

The changes in the peripheral blood reflected those in the bone marrow. In general, when the erythroid elements in the marrow were retarded in maturation, a slowly developing anemia became evident. Repeated examination of blood films indicated this anemia to be normocytic and normochromic in type. In two instances when the red cells appeared macrocytic, determination of absolute cellular constants yielded normal values. Leucopenia in the peripheral blood accompanied a diminution in the more mature granulocytic precursors in the marrow. Thrombocytopenia occurred as a terminal event in two animals, in both of which evidence of tissue hemorrhage was found post mortem. Although the marrow of these latter pigs contained a few megakaryocytes, it seemed possible that some of the large double-nucleated reticulum cells, also present in considerable number, might represent very early forms of platelet precursors.

In the animals which developed leucopenia, there was a marked diminution in the number of circulating lymphocytes as well as neutrophiles. This finding, together with the histologic evidence of proliferation of the endothelial elements in the splenic pulp, suggests that 4-amino PGA may exert a general effect on reticuloendothelial tissue. The advanced degree of fatty change in the liver cells observed in one of the animals which died from the effects of the drug suggests that it also may have a systemic toxic action.

It was found in two animals (Guinea pigs 14 and 15) that a large single dose of as much as 20 to 40 mg. could be administered without producing any hematologic abnormality in the peripheral blood. Moreover, in animals in which prolonged daily dosage had brought about a marked retardation of hematopoietic output, signs of a return toward normal blood formation were evident in the marrow within twenty-four hours after administration was stopped. These two results suggest that in guinea pigs 4-amino PGA is excreted rapidly and that its action is dependent upon the maintenance of a sufficient concentration in the body tissues. Furthermore, unless the animals were moribund, hematologic effects produced by the drug appeared to be completely reversible merely by stopping dosage. In one animal a previous attempt to reverse the changes by the administration of PGA and liver extract was unsuccessful.

An-Fol-R, An-Fol-A, and 7 Methyl Pteroic Acid.—These three compounds were all given over prolonged periods, in doses as large as 20 mg. daily, with remarkably little hematologic effect. Apart from a mild continued reticulocytosis in all instances and a persistent elevation of the leucocyte count in the two animals given 7 methyl pteroic acid, and the one given An-Fol-A, no general or hematologic changes were observed.

Combined Dosage With 4-Amino PGA and Other Antagonists.—In the series of animals in which the effects of combined dosage were investigated, the amount of 4-amino PGA given was chosen as 0.25 mg. daily, a dose which had been shown previously to produce minimal changes over a long period. To this were added the same amounts of An-Fol-R, An-Fol-A, and 7 methyl pteroic acid which had appeared relatively inactive when given alone. The object was to determine whether these three compounds would have any potentiating effect on the action of 4-amino PGA.

4-amino PGA and An-Fol-R, given simultaneously to five animals, produced anemia and leucopenia in all of them, with one death. 4-amino PGA and An-Fol-A caused a moderate degree of anemia and leucopenia in two animals, both of which died. 4-amino PGA and 7 methyl pteroic acid produced anemia with profound leucopenia and terminal thrombocytopenia, killing all three animals observed. In general, these changes occurred in a distinctly shorter period than was required to produce demonstrable effects by the use of 4-amino PGA alone, but the resulting marrow changes were in every way similar to those observed with the latter drug. While this series of experiments is too small to allow definite conclusions to be drawn, it does suggest that An-Fol-R, An-Fol-A, and 7 methyl pteroic acid are themselves true, though weak, depressants of hematopoiesis.

The nature of this investigation does not permit speculation as to the biochemical action of the PGA antagonist compounds. However, on the basis of the cytologic effects, it seems permissible to consider briefly the mechanism whereby the hematologic changes were brought about. The actions of the four antagonists studied are probably similar, though 4-amino PGA is by far the most powerful agent.

The action in guinea pigs would seem to be a generalized slowing of maturation of all blood cell precursors found in normal marrow. The effects may be manifested mainly on one or more of the series of precursors and tend to result in the production of an ever-increasing number of early forms and reticulum cells. Thus, while the marrow becomes more and more filled with primitive cells, anemia, leucopenia, or thrombocytopenia may develop in the peripheral blood before a terminal pancytopenia occurs. These changes are quickly and spontaneously reversible unless dosage with the drugs is maintained.

IV. SUMMARY

1. The hematologic effects of prolonged administration of pteroyl aspartic acid (R and A), 7 methyl pteroic acid, and 4-amino pteroyl glutamic acid to a group of twenty-five guinea pigs are described in detail.

2. 4-amino PGA was found to be the most active compound in depressing hematopoiesis with the production of immature cell types in the bone marrow, and anemia and/or leucopenia in the peripheral blood.

3. The other antagonists studied produced little hematologic effect when administered alone, but each appeared capable of potentiating the action of minimal dosage of 4-amino PGA when given simultaneously.

4. The marrow changes produced were found to be rapidly and spontaneously reversible unless dosage with the compounds was maintained.

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THE EFFECTS OF NITROGEN MUSTARD ON INDUCED ERYTHROBLASTIC HYPERPLASIA IN RABBITS

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INTRODUCTION

THE successful therapy of various benign or neoplastic disease processes with ionizing radiation or chemicals depends to some extent on the selective sensitivity of certain offending cell types. Occasionally, as in the treatment of Hodgkin's disease with the nitrogen mustards, no unusual sensitivity of the Hodgkin's tissue appears to obtain as compared with the normal hematopoietic marrow.[1] It would appear that a significant clinical effect is related to a comparable destruction or depression of normal and neoplastic hematopoietic processes but with a more rapid recovery in the former. This problem of selective and sustained depression of one cell type as opposed to others in the hematopoietic system is well demonstrated in polycythemia rubra vera where hematologic remissions of several years may be induced by a single dose of radiophosphorus[2] or a single course of nitrogen mustards.[3] A serious limitation in the use of a drug such as the nitrogen mustards (methyl-*bis*-(β-chloroethyl) amine hydrochloride) is related to the fact that in some instances the neoplastic process recurs more rapidly than the recovery of the normal hematopoietic tissue, thus making further adequate therapy dangerous or impossible.[4] Likewise the failure of the skin or other normal structures of the body to recover rapidly enough if at all from the absorption of ionizing radiation primarily directed to underlying or adjacent neoplasm imposes the same restriction. These remarks only serve to indicate the importance of determining ways and means of rendering normal cells of the body temporarily more resistant to the therapeutic agent than the cell population of the neoplasm. It was reported recently by Jacobson and associates[5, 6, 7] that the erythrocytopoietic tissue of the rabbit could be protected from the destruction of x-irradiation if the erythropoietic tissue were previously stimulated by the withdrawal of blood or the administration of phenylhydrazine. Since the biologic effects of ionizing radiations and methyl-*bis*-(β-chloroethyl) amine hydrochloride† are so similar, especially as they effect the hematopoietic system,[2, 8] it was decided to determine whether stimulation of erythrocytopoiesis would similarly reduce the degree of destruction produced by the latter chemical agent. This communication relates these studies. Certain studies with x-irradiation carried on simultaneously are included for comparative purposes.

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†Hereafter referred to as HN_2 or nitrogen mustard.

MATERIALS AND METHODS

Swift's snuffle-free rabbits of uniform age and weight (2½ to 3 kilograms) were used exclusively in this study. The rabbits were divided into four groups (Table I). Group 1 consisted of normal, untreated animals; Group 2 received 3 mg. of nitrogen mustard intravenously per kilogram of body weight; Group 3 consisted of rabbits with a phenylhydrazine-induced anemia, and Group 4 received 3 mg. of nitrogen mustard intravenously after an anemia had been induced by phenylhydrazine.

TABLE I. NUMBER OF ANIMALS AND TREATMENT OF VARIOUS GROUPS. HEMATOLOGIC STUDIES

GROUP	PHENYLHYDRAZINE	NITROGEN MUSTARD	NUMBER OF ANIMALS
1	–	–	17
2	–	3 mg./kg.	19
3	40 mg.	–	10
4	40 mg.	3 mg./kg.	37

In preliminary experiments it was found that 3.0 mg. of nitrogen mustard per kilogram of body weight was approximately the LD_{50}/14-day dose for rabbits. This means that 3 mg. per kilogram were sufficient to kill 50 per cent of the rabbits within fourteen days. On the other hand, 800 r of x-radiation is the LD_{50}/30-day dose in our laboratory for this strain of rabbit.[9] No adequate criterion exists on which to base the comparability of these totally different agents. Since the histologic damage in the marrow present at three days was more or less comparable after 3 mg. per kilogram of nitrogen mustard or 800 r whole body x-irradiation, these doses were arbitrarily chosen as equivalent doses for the purposes of this study.

Technique of Nitrogen Mustard Administration.—Three milligrams of nitrogen mustard per kilogram of body weight were slowly injected into the marginal ear vein of the rabbit immediately after the chemical had been dissolved in physiologic saline. A concentration of 1 mg. of the drug per 1 c.c. of saline was used since this concentration produced no sloughing at the site of injection and did not obliterate the ear vein.

Technique of X-Irradiation.—The x-rays administered in these experiments were generated on a 200 KVP machine operating at 15 milliamperes. A 0.5 mm. copper filter and a 1 mm. aluminum filter were used. The half-value layer in copper of the filtered beam was 0.98 millimeter. The exposure was measured with a Victoreen condenser r-meter equipped with a 100 r chamber. Measurements were made in air within a treatment box at a position occupied by the center of the animal's body. The exposure distance was approximately 30.5 inches. Victoreen chambers of 250 r full scale readings were used as monitors.

Hematologic Studies.—The studies on the peripheral blood taken from the rabbit, using standard techniques, included the following:

(a) Erythrocytes per cubic millimeter.
(b) Hemoglobin in grams per 100 cubic centimeters.
(c) Reticulocytes in per cent of erythrocytes.
(d) Total leucocytes per cubic millimeter.
(e) Differential leucocytes.

Studies on blood obtained by cardiocentesis (venous or arterial blood) included:

(f) Platelet counts.
(g) Whole blood clotting time (Lee-White).
(h) Prothrombin determinations.
(i) Heparin tolerance test.
(j) Fibrinogen and calcium determinations.

These latter studies on the effect of nitrogen mustard on coagulation of blood have been reported in detail elsewhere[10] and will, therefore, not be related here.

Histopathologic Studies.—Histologic studies were made in order to study the blood-forming tissues for the proper correlation with the peripheral blood on control animals, animals given HN_2 only, animals given phenylhydrazine, and animals given phenylhydrazine

followed by nitrogen mustard. Animals were sacrificed at frequent intervals the first nine days and at less frequent intervals thereafter; the tissues were fixed in Zenker-formol, embedded in nitrocellulose, cut at 8 or 6 μ, and stained with hematoxylin-eosin-azure II. (Table II.)

TABLE II. HISTOPATHOLOGIC STUDIES SHOWING NUMBER OF ANIMALS SACRIFICED AT VARIOUS INTERVALS

TIME AFTER NITROGEN MUSTARD INJECTIONS	CONTROL RABBITS (UNTREATED)	RABBITS GIVEN NITROGEN MUSTARD (3 MG./KG.)	RABBITS TREATED WITH PHENYLHYDRAZINE (40 MG. S.C.)	RABBITS TREATED WITH PHENYLHYDRAZINE AND NITROGEN MUSTARD
0 hr.*	1	0	1	0
1 hr.	0	1	0	0
3 hr.	1	1	1	2
9 hr.	0	1	1	0
12 hr.	0	1	1	2
1 day	1	2	1	2
2 days	0	2	1	2
3 days	1	3	1	2
4 days	0	2	1	3
5 days	0	2	1	2
6 days	0	2	0	2
7 days	1	2	1	2
8 days	1	0	0	0
9 days	0	2	0	2
12 days	1	0	0	2
14 days	1	1	1	2
16 days	0	1	1	0
21 days	0	0	2	1
35 days	1	0	2	2

*Two days after phenylhydrazine withdrawal.

RESULTS

Mortality Studies.—The mortality rate of the animals in Group 4 which received nitrogen mustard and phenylhydrazine was slightly higher than that of the animals of Group 2 which received mustard alone. The survival data indicate that 3 mg. of nitrogen mustard injected intravenously kills approximately 50 per cent of the animals in fourteen days. The most critical period appears to be the fourth day. However, no experiments involving large numbers of animals were undertaken to determine the LD_{50} dose and, therefore, the 3 mg. per kilogram dose can be considered only a tentative and approximate LD_{50} value.

Hematologic Findings.—

Erythrocyte Values: The erythrocyte values of the animals in Groups 3 and 4 that received phenylhydrazine were reduced to 3.3×10^6 and 3.5×10^6 respectively at time O (Fig. 1). The animals in Group 3 which received phenylhydrazine alone recovered from the anemia in twelve days. The erythrocyte values of the rabbits which received nitrogen mustard after the anemia had been produced (Group 4) continued to fall, reaching a mean minimum value of 2.8×10^6 by the third day after the mustard injection; they remained thus depressed through five days, then gradually reached normal values by the twelfth day and remained within normal limits throughout the balance of the experimental period (thirty-five days). Between the sixth and twenty-second day a decrease (circa 15 per cent) in the erythrocyte values occurred in the Group 2 rabbits which received nitrogen mustard alone.

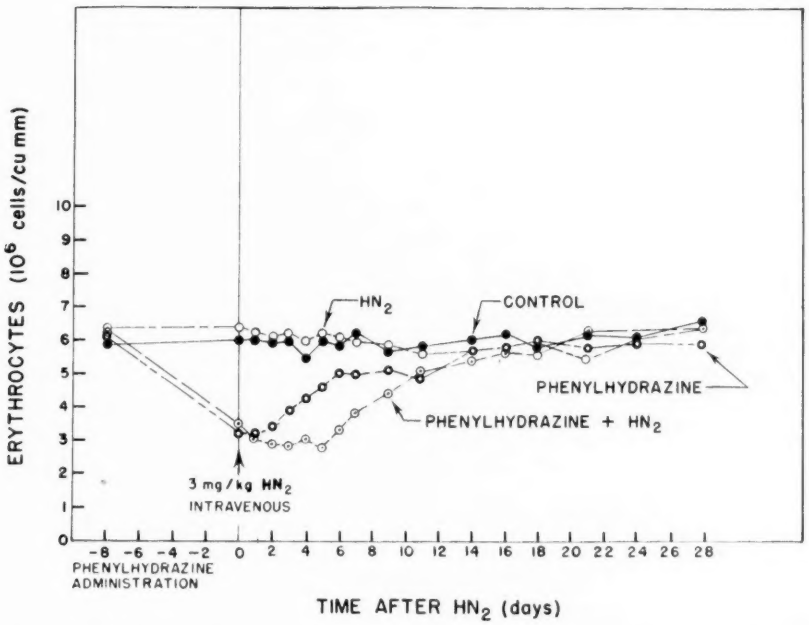


Fig. 1.—The effect of nitrogen mustard in a single intravenous dose of 3 mg. per kilogram on the erythrocyte values of control rabbits and rabbits injected with phenylhydrazine prior to the administratiin of nitrogen mustard.

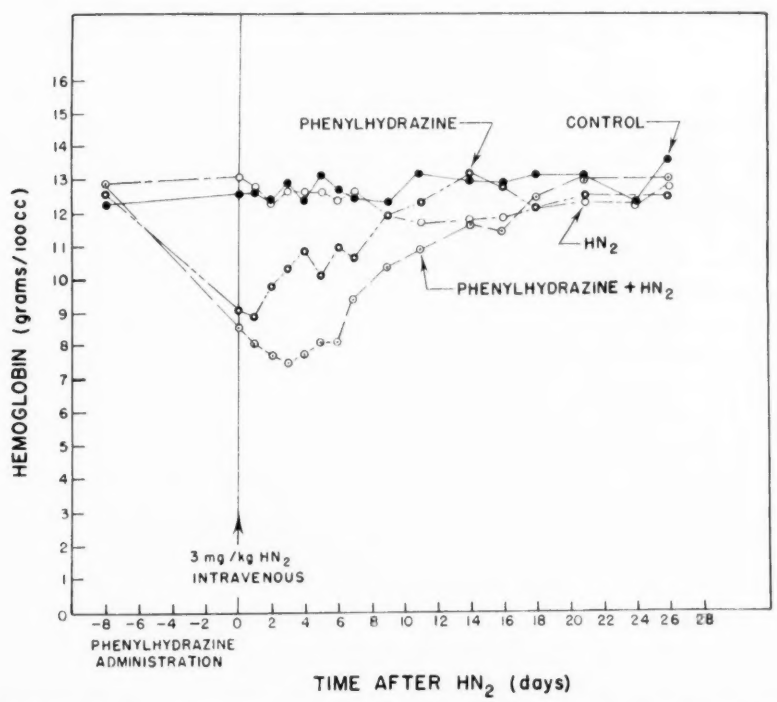


Fig. 2.—The effect of nitrogen mustard in a single intravenous dose of 3 mg. per kilogram on the hemoglobin values of control rabbits and rabbits injected with phenylhydrazine prior to the administration of nitrogen mustard.

Hemoglobin: The hemoglobin values (Fig. 2) of the various groups followed a pattern of response comparable with that described for the erythrocytes.

Reticulocytes: The mean reticulocyte value in the control group (Group 1) varied between 3.2 and 4.2 per cent (Fig. 3). The mean reticulocyte value of Groups 3 and 4 rose as a result of the phenylhydrazine administration to 22.6 and 25.4 per cent respectively. The mean reticulocyte value of Group 3 fell rapidly after the phenylhydrazine was discontinued from a maximum of 22.6 to 4.3 per cent and remained essentially in the normal range thereafter. The reticulocyte values of Group 2 which received nitrogen mustard only fell to 0.2 per cent in three days, remained below these values through the fifth day, then gradually rose to the normal range by the ninth day. The reticulocytes of the Group 4 animals, on the other hand, which received mustard after the phenylhydrazine-induced anemia fell abruptly to a minimum of 1.6 per cent by the third day, rose rapidly to 15.6 per cent by the sixth day, then gradually fell to normal by the sixteenth day.

Leucocytes: The mean leucocyte value of the rabbits which received nitrogen mustard only (Group 2) and those that received mustard after the phenylhydrazine-induced anemia (Group 4) fell to circa 600 per cubic millimeter by the third day (Fig. 4). The mean leucocyte value of the animals that received mustard alone (Group 2) continued to fall, reaching a minimum by the sixth day, after which recovery occurred by the ninth day. The leucocyte value of the animals that received nitrogen mustard after the phenylhydrazine-induced anemia (Group 4) gradually rose after the third day to an essentially normal value by the ninth day. An initial rise in the absolute heterophil value above the normal occurred in twenty-four hours in both Groups 2 and 4. In three days the heterophil value fell maximally in Group 4 animals which had received phenylhydrazine and nitrogen mustard, whereas the heterophils in the Group 2 rabbits that received mustard alone reached a minimum value in five days (Fig. 5). An abortive rise occurred in both groups on the fifth and sixth days respectively. Both groups returned to normal values by the ninth day and remained essentially normal thereafter.

The absolute number of lymphocytes per cubic millimeter in Groups 2 and 4 paralleled the leucocyte curve closely (Fig. 6). Partial recovery had occurred by the ninth day, but complete recovery had not occurred in thirty-five days.

The leucocyte values, including the heterophil and lymphocyte values of the animals with the phenylhydrazine-induced anemia only (Group 3), were slightly depressed by the fifth day, remained thus depressed for circa six days, rose above normal values by the sixteenth day, returned to normal values by the eighteenth day, and remained within normal limits thereafter (Figs. 4, 5, and 6).

Histologic Studies.—

General Observations on Ectopic Blood Formation in Lymphatic Tissue: Scattered about the red pulp in the spleen of normal animals a few small islands

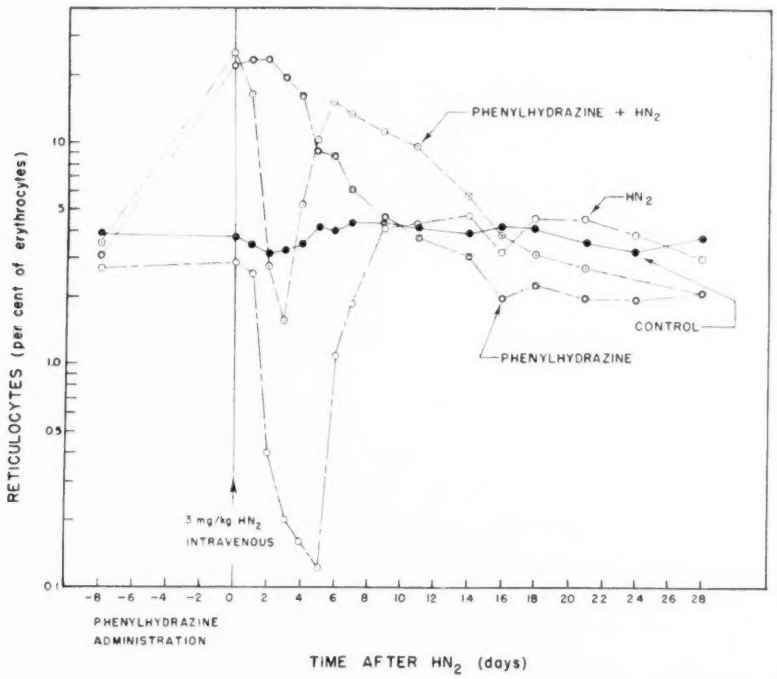


Fig. 3.—The effect of nitrogen mustard in a single intravenous dose of 3 mg. per kilogram on the reticulocyte values of control rabbits and rabbits injected with phenylhydrazine prior to the administration of nitrogen mustard.

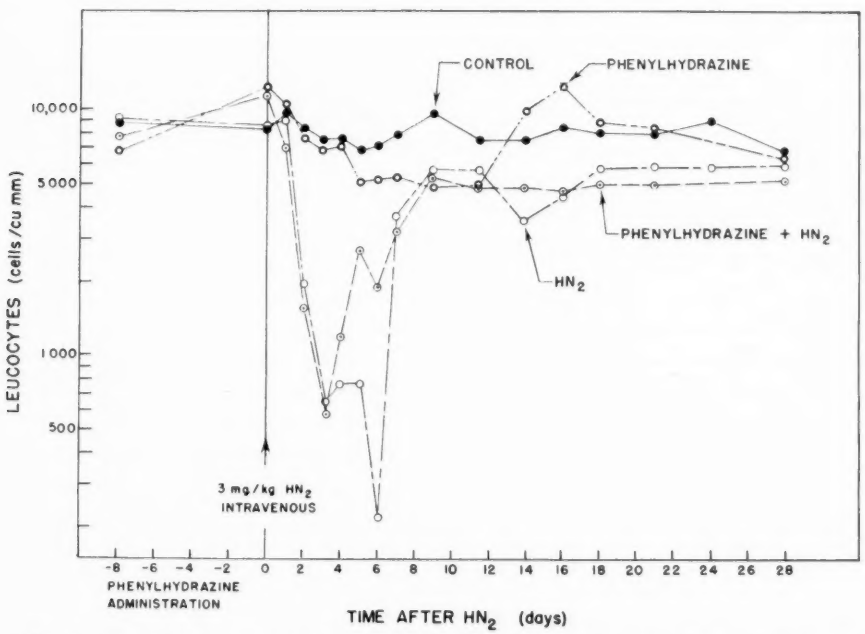


Fig. 4.—The effect of nitrogen mustard in a single intravenous dose of 3 mg. per kilogram on the leucocyte values of control rabbits and rabbits injected with phenylhydrazine prior to the administration of nitrogen mustard.

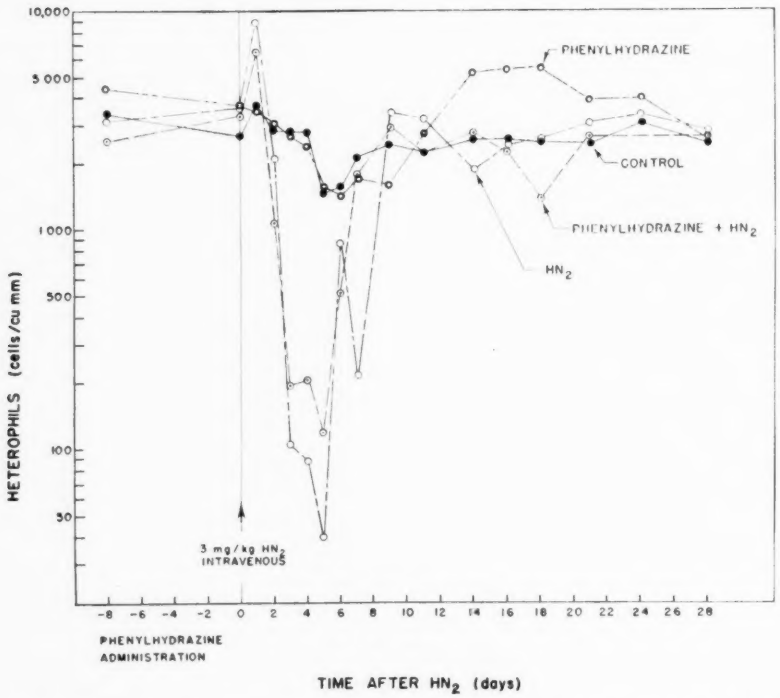


Fig. 5.—The effect of nitrogen mustard in a single intravenous dose of 3 mg. per kilogram on the heterophil values of control rabbits injected with phenylhydrazine prior to the administration of nitrogen mustard.

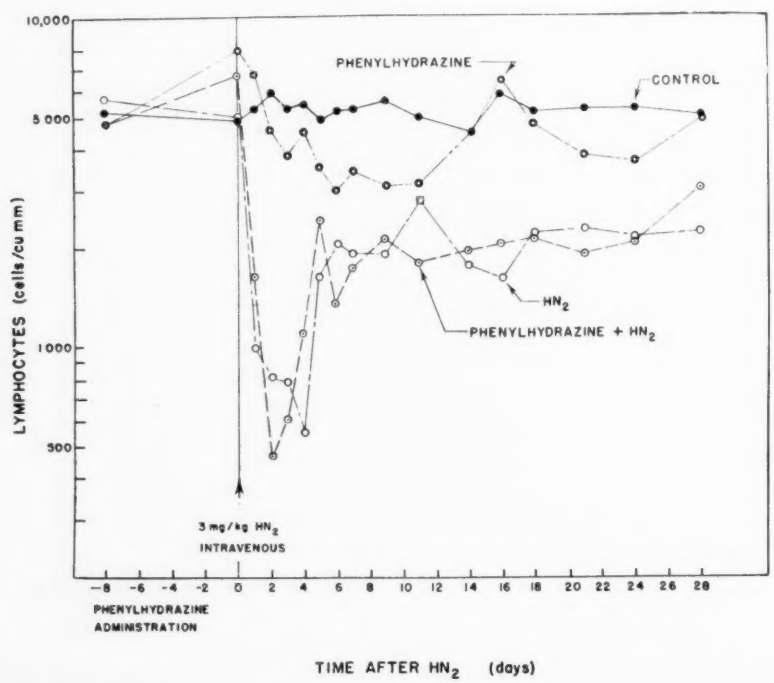


Fig. 6.—The effect of nitrogen mustard in a single intravenous dose of 3 mg. per kilogram on the lymphocyte values of control rabbits and rabbits injected with phenylhydrazine prior to the administration of nitrogen mustard.

of ectopic erythrocytopoiesis and granulocytopoiesis and an occasional megakaryocyte can be found. At the height of the hyperplastic reaction in the bone marrow (two days after last dose of phenylhydrazine) the amount of this ectopic blood formation in the spleen is increased by at least 100 per cent. These islands gradually decrease in extent over a period of three weeks following the last dose of phenylhydrazine, leaving only the normal amount seen in most of the young adult rabbits. Ectopic blood formation in lymphatic tissue elsewhere in the body was not increased appreciably by phenylhydrazine.

Effect of Nitrogen Mustard on Lymphatic Tissue.—The effect of HN_2 in the dose employed on the lymphatic tissue of the thymus, mesenteric node, and spleen will only be summarized. An intense degeneration of lymphocytes was apparent within the first twenty-four hour period. In fact, six hours after injection of HN_2 lymphocytes were in all stages of disintegration in the lymph nodes, thymus, and spleen. By the twelfth hour the degeneration phase was very marked and many macrophages within the nodules and sinuses were filled with particulate debris. Mitosis in lymphocytes was not seen at the twenty-four hour stage. Twenty-four hours after injection the debris-laden phagocytes were almost entirely gone except in the nodules of the spleen, lymph nodes, and thymus, and only minimal evidence of continued cell death was evident. At the twenty-four hour stage however, there was a marked heterophil invasion of the lymph node, red pulp of the spleen, and of the thymus to a lesser degree. Two days after HN_2 injection the lymphocytes of the spleen had practically disappeared except for a few small to medium lymphocytes about the central arterioles of the nodules. The lymphocyte population of the lymph nodes and thymus, though markedly depleted, was so to a lesser degree than that of the spleens. Only a few granulocytes scattered throughout the red pulp were found in these lymphatic structures at this stage. The atrophic phase lasted through the fifth day in these lymphatic tissues. During this period little evidence of regeneration was evident. Only occasional mitoses were seen in the lymphocytes. From the sixth day stage on, however, the amount of lymphatic tissue in the spleen, lymph node, and thymus slowly increased. Mitoses were again more prominent. Not until about the fourteenth day had the histologic reconstitution of lymphatic tissue reached the state which made differentiation from the normal control difficult if not impossible.

The effect of a single injection of nitrogen mustard on ectopic erythropoiesis and granulocytopoiesis in the spleen was difficult to assess in terms of any differential sensitivity or insensitivity because of the small amount of such tissue present in the spleen. There was, however, a definite decrease in amount of ectopic erythrocytopoiesis and granulocytopoiesis following HN_2 injection.

Bone Marrow.—

(a) Femoral Bone Marrow of Normal Rabbit (Group 1) and Rabbits With Hyperplastic Marrow (Two Days After Last Phenylhydrazine Injection): The femoral marrow of the normal control rabbits (Group 1) used in this study was composed of roughly 60 per cent fat. The balance of the marrow was composed of hematopoietic tissue. The free cells were predominantly granulo- and erythrocytopoietic but contained scattered hemocytoblasts, megakaryocytes, and

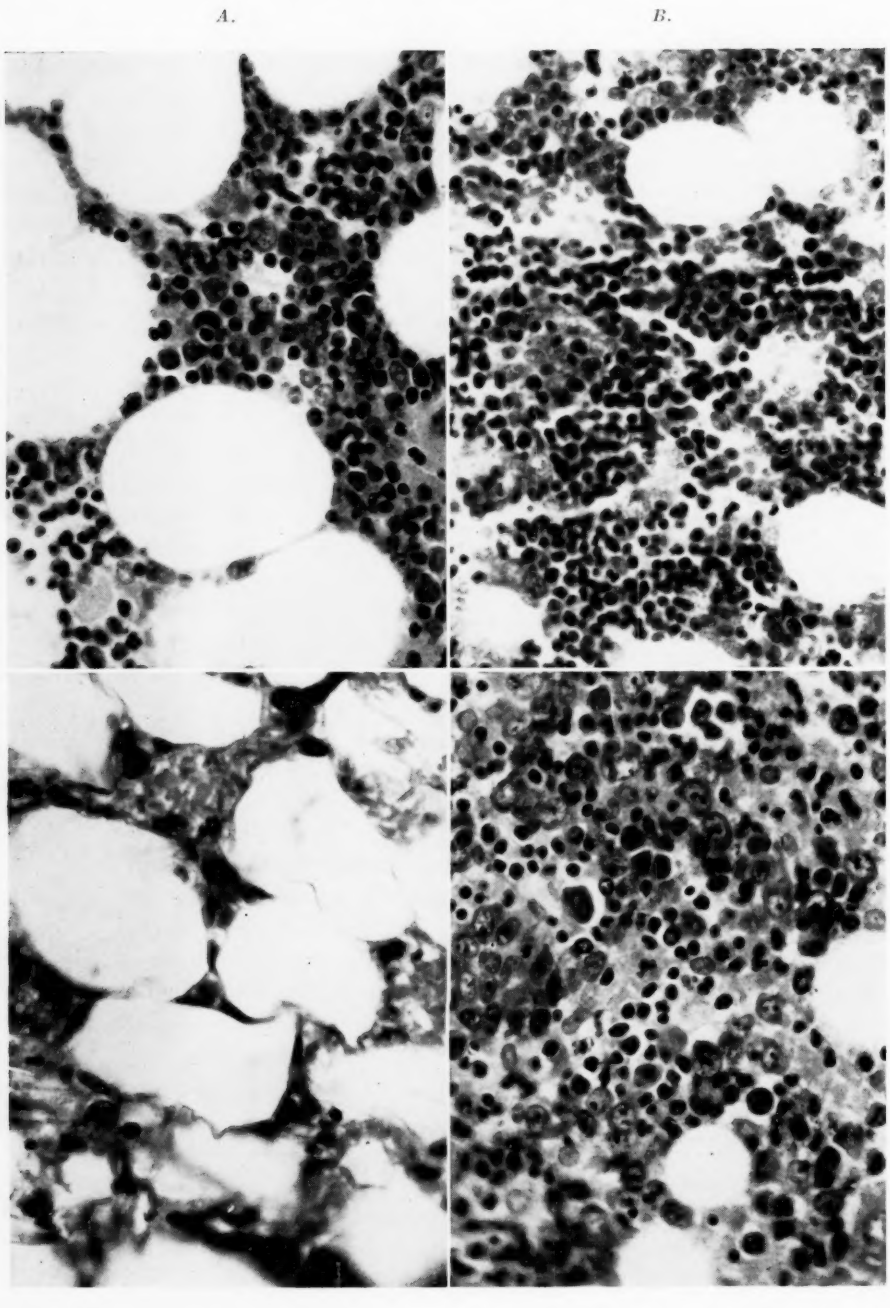


Fig. 7.—The effect of nitrogen mustard on the femoral marrow of normal rabbits and on rabbits which had previously received phenylhydrazine (×420).

A, Normal.

B, Four days after last injection of phenylhydrazine. Note the general hyperplasia and reduction in size of fat spaces.

C, Two days after intravenous injection of nitrogen mustard in a dose of 3 mg. per kilogram. The space normally occupied by marrow cells is filled with a homogeneous basophilic-staining material and the general cellularity is drastically reduced.

D, Two days after intravenous injection of nitrogen mustard (3 mg. per kilogram) and four days after phenylhydrazine withdrawal. Note that fat spaces are small; the general cellularity is greater than normal; hemocytoblasts and basophilic erythroblasts are abundant and larger than normal.

plasma cells. Only relatively few proerythroblasts (basophilic erythroblasts) were found. Mitoses were seen in granulocytes, erythrocytes, and megakaryocytes but were not prominent. In general, this active hematopoietic tissue was largely concentrated in the metaphysis and along the endosteum of the entire diaphysis, diminishing toward the center of the diaphysis. The variation in the activity of the normal marrow is shown in Figs. 7, 8, and 9.

The femoral marrow of rabbits two days after the last dose of phenylhydrazine (Group 3) was extremely hyperplastic. The hematopoietic marrow occupied from 85 to 95 per cent of the marrow space. Fat was correspondingly decreased. This hyperplastic marrow consisted chiefly of an increase in hemocytoblasts (Downey's myeloblast) and erythroblasts in all stages of maturation. The increase in basophilic erythroblasts was particularly conspicuous. Mitoses in these latter cells were numerous. A modest increase of granulocyte percursors and megakaryocytes was also apparent. The marrow was replete with all stages of transformation of reticular cells to erythroblasts or hemocytoblasts. The marrow hyperplasia reached its maximum two days after withdrawal of phenylhydrazine, paralleling the maximum reticulocyte response in the peripheral blood of this group (Fig. 3). Only gradually over a period of approximately two weeks did the bone marrow return to a normal state of activity after withdrawal of phenylhydrazine. Figs. 7, 8, 9, and 10 illustrate the marrow hyperplasia and the lessening of hyperplasia following phenylhydrazine withdrawal. As the erythroblastic hyperplasia in the bone marrow increased, the amount of phagocytized iron increased, but was evident also in the later intervals after phenylhydrazine withdrawal as the marrow returned to a normal state. As indicated previously, it was at the height of the hyperplasia (two days after last injection of phenylhydrazine) that HN_2 was given to the rabbits in Group 4. Animals in Group 2 (normal, previously untreated animals) were given HN_2 simultaneously.

(b) Effect of HN_2 on Normal Animals (Group 2) and Animals With Hyperplastic Marrow (Group 4). Period of Degeneration (Zero to Twenty-four Hours Following HN_2 Injection): Degeneration (pyknosis, karyorrhexis, and phagocytosis) was apparent in both the granulopoietic and erythropoietic cells as early as six hours after the injection of HN_2 in both Group 2 (normal animals which received HN_2) and Group 4 (animals which received HN_2 after a phenylhydrazine-induced anemia), but the intensity of this degeneration was increased at the twelve-hour stage. Phagocytosis of debris in the marrow was marked at the twelve-hour stage. Destruction of megakaryocytes was not as obvious. Some megakaryocytes were in a stage of disintegration in twenty-four hours. The destruction of the more mature erythroblasts was more rapid than the destruction of granulocytes. In twenty-four hours the cells of the marrow of the normal rabbits which received HN_2 (Group 2) were largely granulopoietic with a few scattered erythroblasts (mostly basophilic), hemocytoblasts, and megakaryocytes.

The marrow of the animals whose marrow was hyperplastic when HN_2 was given (Group 4) contained granulocytes, scattered megakaryocytes, and numerous nemocytoblasts and basophilic erythroblasts at this twenty-four hour

stage. Again transformation of reticular cells to erythroblasts and hemocytoblasts was conspicuous. Mitoses were absent in the marrow of the animals which received HN_2 only (Group 2) in six hours and none were seen at subsequent intervals studied through twenty-four hours. Mitoses were present in all the intervals studied during this destructive phase in the animals which had phenylhydrazine and HN_2 (Group 4). Mitoses were largely confined to the erythroblasts. They were fewer in number than in the animals that received phenylhydrazine only (Group 3) at a comparable stage, but at least 200 per cent higher than in normal untreated controls (Group 1). Little basophilic-staining "gelatinous" marrow was evident in animals which received HN_2 only (Group 2) at the twenty-four hour interval. In the phenylhydrazine and HN_2 animals (Group 4), however, gelatinous marrow as well as dilated vessels were prominent, but the residual cellularity of the latter was much greater.

Period of Atrophy (Twenty-Four Hours Through Four Days).—The marrow of animals which received HN_2 only (Group 2) was almost completely devoid of cells on the second, third, and fourth day intervals and consisted of dilated vessels, some gelatinous material, scattered reticular cells, unidentified mononuclear cells, occasional normal or giant erythroblasts, hemocytoblasts, megakaryocytes, normal or giant myelocytes, plasma cells, and fat cells. Only rarely were mitotic figures observed during this period. This extreme atrophy is illustrated in Figs. 7 and 8.

The marrow of animals which received phenylhydrazine and HN_2 (Group 4) contained a large amount of basophilic-staining gelatinous material, dilated vessels and phagocytized debris. Large islands of hyperplastic marrow were scattered about and were composed almost exclusively of hemocytoblasts and basophilic erythroblasts two days after HN_2 injection. Other areas contained many of these precursors as well as more mature erythroblasts (Fig. 7, *D*). The amount of residual granulopoietic and megakaryocytopoietic cells was greater than in the marrow of animals at a corresponding interval which received HN_2 only (Group 2). Many giant myelocytes and erythroblasts were seen scattered about as were clover-leaf patterns in the erythroblasts. There was a questionable increase in the number of plasma cells. The surviving hemocytoblasts and basophilic erythroblasts overwhelmed the other cell types in numbers and many parts of the marrow were composed exclusively of one or the other or both. The size of these cells as compared with similar cells in the marrow of the other groups (1, 2, and 3) is illustrated in Figs. 7, 8, and 9. The amount of erythrocytopoietic marrow on the second day was estimated to be at least 200 per cent greater than normal marrow (Group 1), 200 to 300 per cent greater on the third day, and 300 to 400 per cent greater on the fourth day. Mitoses were very prominent in the erythroblasts at these intervals. Granulopoietic and megakaryocytopoietic cells and the mitotic activity of these cells increased slowly at first but were relatively quite prominent by the fourth day. As was evident during the period of destruction (zero to twenty-four hours), transformation of reticular cells to erythroblasts and hemocytoblasts was prominent. In fact, direct transformation of reticular cells to giant myelocytes was becoming decidedly more prominent during the third and fourth days after HN_2 in this group (Group 4).

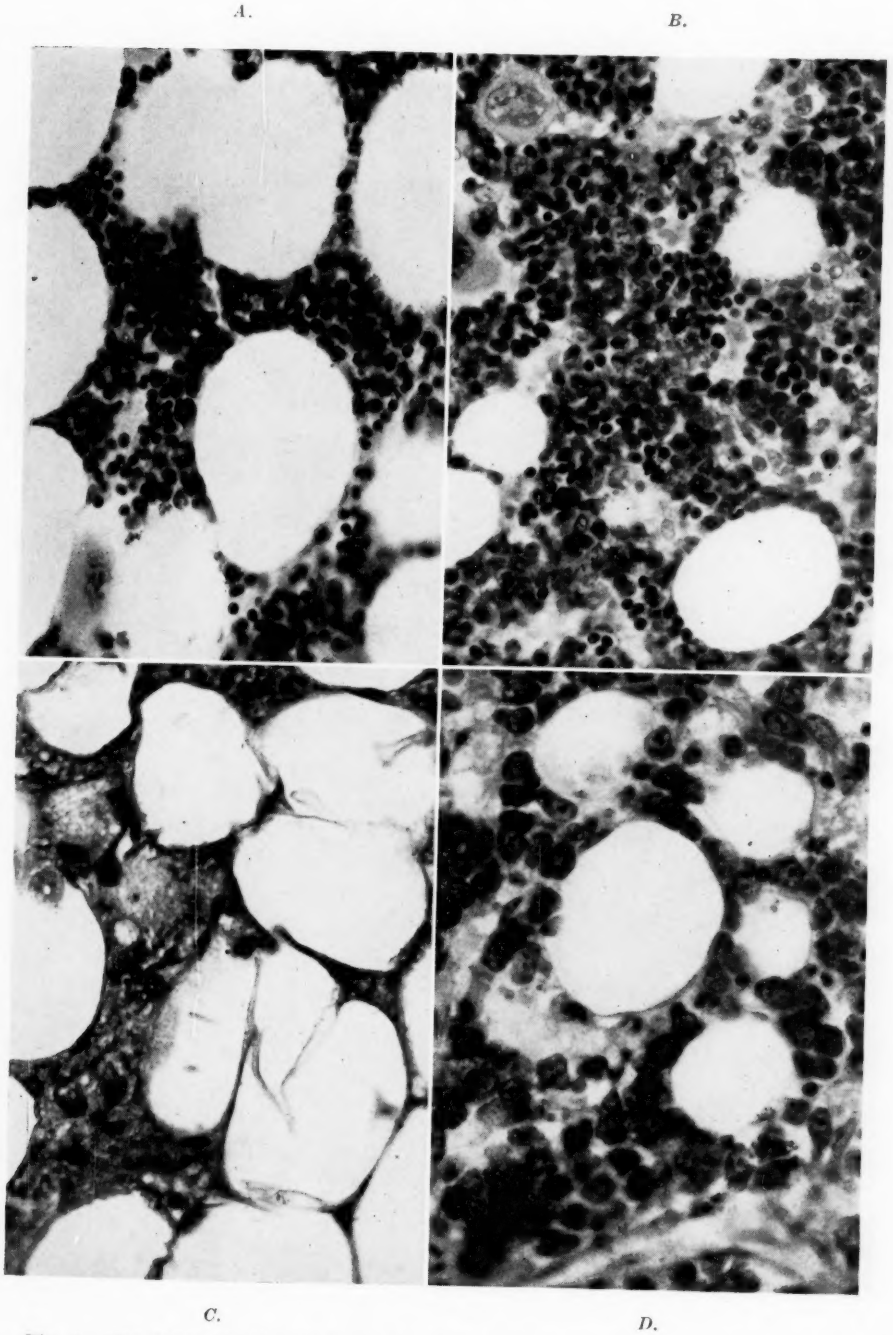


Fig. 8.—The effect of nitrogen mustard on the femoral marrow of normal rabbits and on rabbits which had previously received phenylhydrazine (×420).

A, Normal.

B, Six days after last injection of phenylhydrazine the general hyperplasia still exists.

C, Four days after intravenous injection of nitrogen mustard in a dose of 3 mg. per kilogram. Cellular depletion marked.

D, Four days after intravenous injection of nitrogen mustard (3 mg. per kilogram) and six days after phenylhydrazine withdrawal. Cell population largely hemocytoblasts and basophilic erythroblasts. Note the size of these precursors as compared with cells in normal marrow.

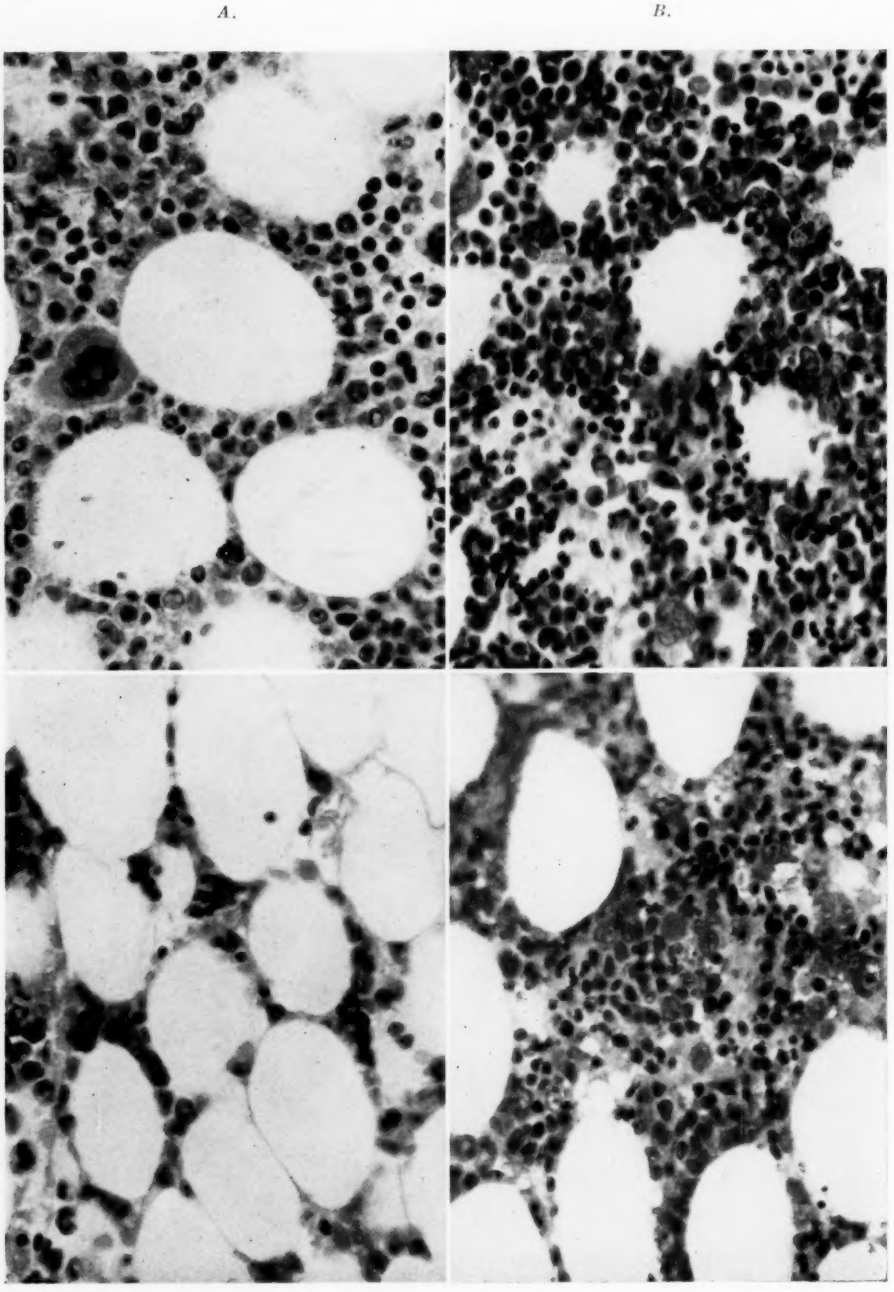


Fig. 9.—The effect of nitrogen mustard on the femoral marrow of normal rabbits and on rabbits which had previously received phenylhydrazine (×420).

A, Normal.

B, Eight days after last injection of phenylhydrazine, showing persistance of hyperplasia which is largely erythropoietic.

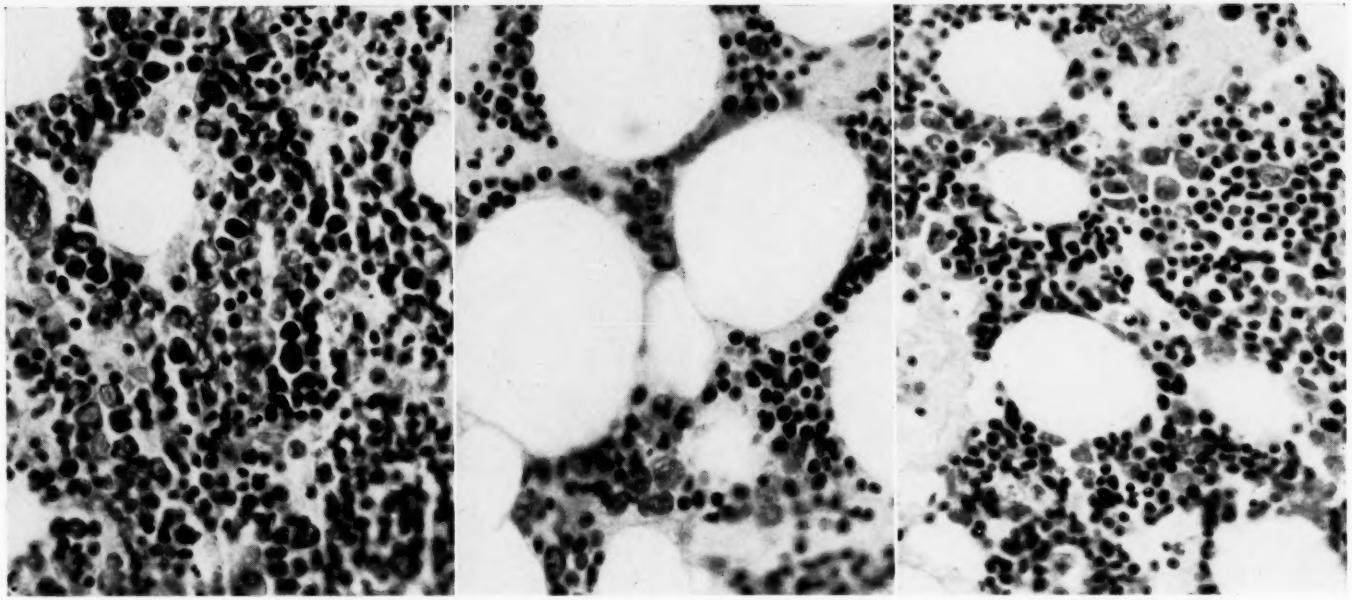
C, Six days after intravenous injection of nitrogen mustard (3 mg. per kilogram). Regeneration is progressing at this stage.

D, Six days after intravenous injection of nitrogen mustard (3 mg. per kilogram) and eight days after phenylhydrazine withdrawal. Hyperplasia involves granulopoietic and erythropoietic series.

Period of Regeneration (Five Days Through Fourteen Days).—The marrow of animals which received HN_2 only (Group 2) contained scattered islands of hemocytoblasts and myelocytes and erythroblasts in various stages of maturation on the fifth day after the HN_2 injection and increasingly greater numbers on the sixth and seventh day intervals. On the eighth and ninth day intervals granulocytes and megakaryocytes became more conspicuous. This more or less orderly regeneration continued; by the fourteenth day the marrow was essentially similar to that of the untreated controls of Group 1. This period of active regeneration is illustrated in Figs. 9 and 10. In the marrow of animals which received phenylhydrazine prior to the HN_2 injection (Group 4), erythroblastic hyperplasia continued to be active far in excess of Group 2 (HN_2 only) or Group 1 (normal) and only gradually returned to a normal state. Whereas evidence of granulo- and megakaryocytopoietic regeneration was conspicuous on the eighth day in the animals that received HN_2 only (Group 2), active regeneration of granulo- and megakaryocytopoietic cells was evident beginning on the fourth day and increased through the ninth day in the animals which received phenylhydrazine and HN_2 (Group 4). The intense erythroblastic proliferation seen at previous intervals continued with only slight diminution during this period.

Comparison of X-ray and HN_2 on Phenylhydrazine-Induced Anemia and Hyperplastic Marrow.—In a previous series of papers[5, 6, 7] the effects of 800 r whole body x-irradiation on the peripheral blood and bone marrow of normal rabbits and rabbits with a phenylhydrazine-induced anemia have been described in detail. The conditions of these experiments were identical with those of the experiments described in this paper except that a physical agent (800 r x-ray) was given instead of a chemical agent (HN_2). Although the main findings in the x-ray experiments are essentially the same as in the HN_2 experiment, certain gross differences make a brief comparison worthwhile. As was demonstrated in Figs. 1 and 2, nitrogen mustard alone produced an anemia which was on the borderline of significance some two weeks or more after the HN_2 administration. Animals which had a phenylhydrazine-induced regenerative anemia developed further anemia within the first five days after HN_2 administration. A dose of 800 r whole body x-irradiation produced an anemia in rabbits which reached a maximum on about the fourteenth day (Fig. 11). On the other hand, the administration of 800 r to animals with a phenylhydrazine-induced regenerative anemia produced neither an early nor a latent anemia and recovery proceeded rapidly but not as rapidly as occurred in the phenylhydrazine and HN_2 animals (Fig. 12). This difference in the early effect of phenylhydrazine followed by HN_2 on the one hand and phenylhydrazine followed by x-irradiation on the other was not apparent in the bone marrow since in neither was erythrocytopoiesis seriously interrupted.

Nitrogen mustard alone and x-ray alone produced a comparable degree of cellular depletion in the marrow of normal rabbits, but bone marrow recovery was more rapid in the HN_2 series than in the x-ray series (Compare Fig. 9, *C* with Fig. 13, *A*). Similarly, the degree of cellular depletion in the marrow



A. *B.* *C.*

Fig. 10.—The effect of nitrogen mustard on the femoral marrow of normal rabbits and on rabbits which had previously received phenylhydrazine (×420).

A, Eleven days after last injection of phenylhydrazine.

B, Nine days after intravenous injection of nitrogen mustard (3 mg. per kilogram). Marrow essentially normal.

C, Nine days after intravenous injection of nitrogen mustard (3 mg. per kilogram) and eleven days after phenylhydrazine withdrawal. Marrow now essentially normal except that general hyperplasia persists.

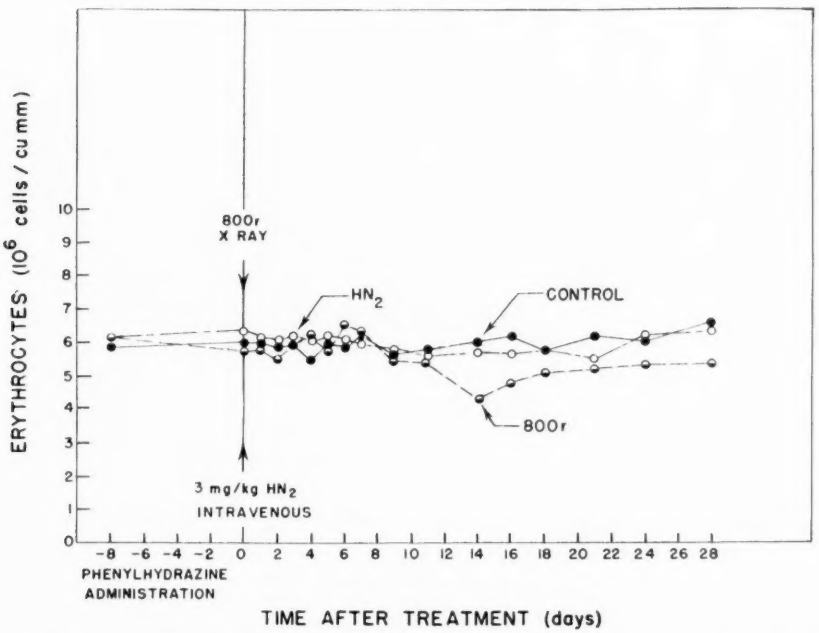


Fig. 11.—Comparative effect of 800 r whole body x-irradiation and 3 mg. per kilogram nitrogen mustard on the circulating erythrocytes of normal rabbits.

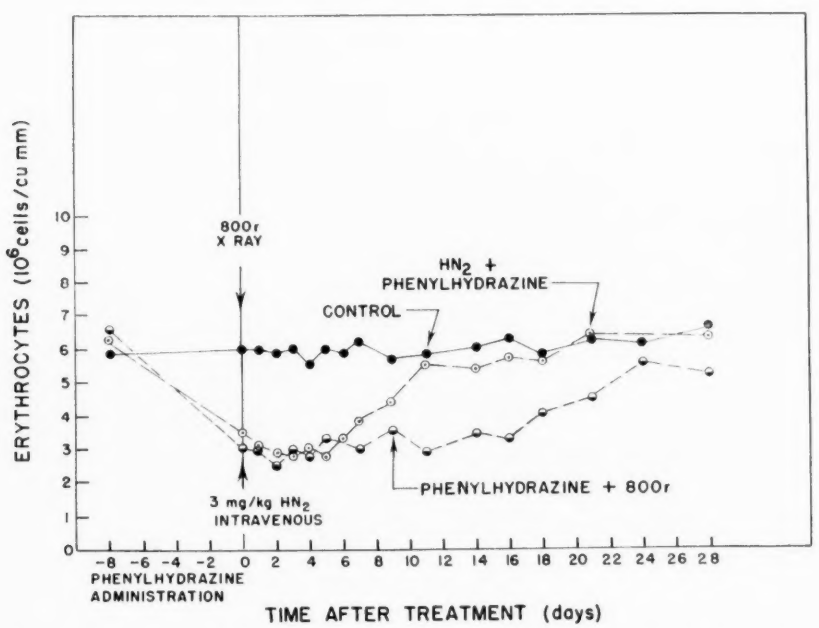
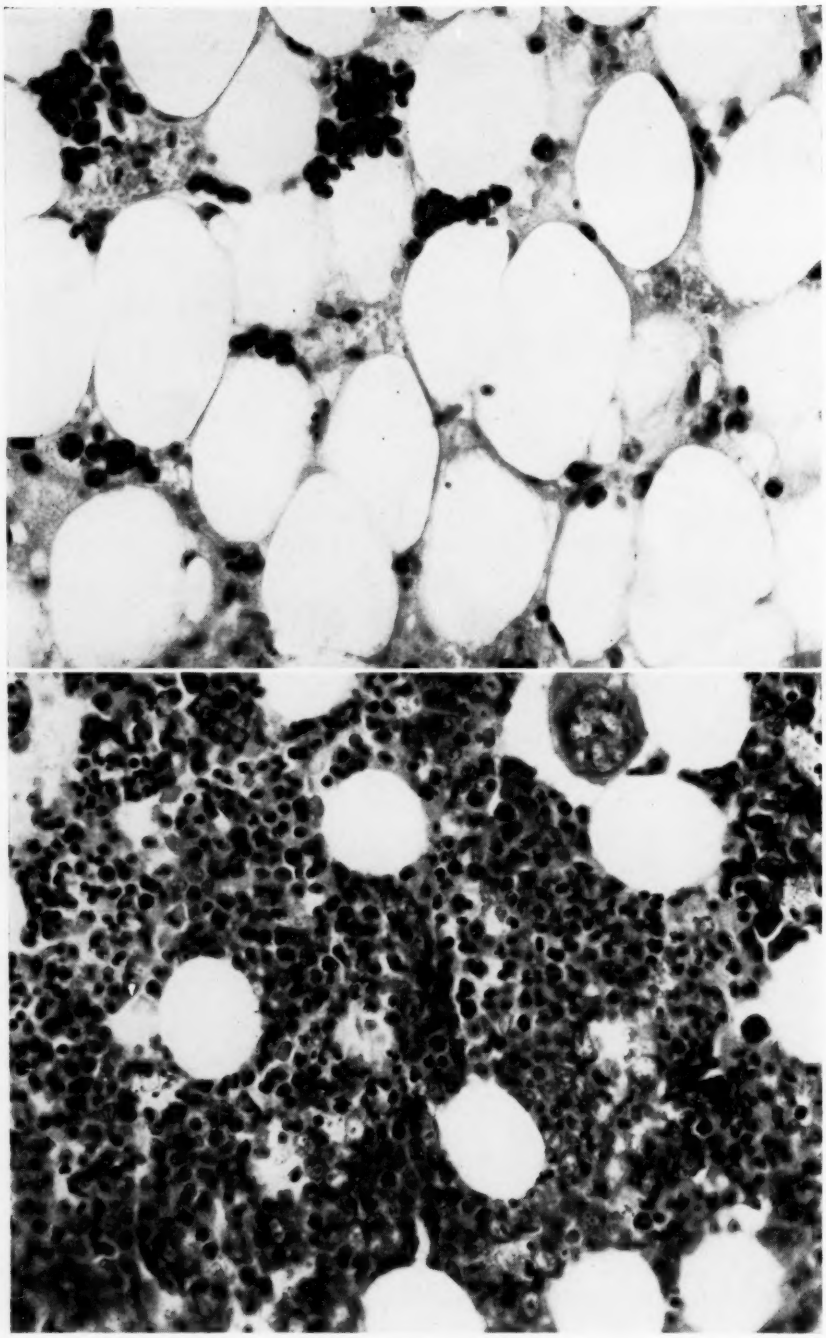


Fig. 12.—Comparative effect of 800 r whole body x-irradiation and nitrogen mustard (3 mg. per kilogram) on the erythrocyte values of rabbits which had previously been made anemic by phenylhydrazine administration.

A.



B.

Fig. 13.—The effect of 800 r whole body x-irradiation on the femoral marrow of a normal rabbit and on rabbits which had previously received phenylhydrazine (×420).

A, Six days after 800 r whole body x-irradiation. Except for small islands of erythrocytopoiesis the marrow is still atrophic.

B, Six days after 800 r whole body x-irradiation and eight days after last injection of phenylhydrazine. This shows the marked hyperplasia which is found scattered about in the marrow.

of animals which received both phenylhydrazine and HN_2 was comparable with the cellular depletion in the bone marrow of animals which received both phenylhydrazine and x-ray in the early stages (one day after HN_2 or x-ray respectively). However, the marrow of the phenylhydrazine-HN_2 animals underwent a more rapid regeneration than that of the phenylhydrazine-x-ray animals.

DISCUSSION

It is interesting that the production of a regenerative anemia with phenylhydrazine or bleeding protects against the usual latent radiation-induced anemia in rabbits by virtue of the survival of sufficient hematopoietic tissue to insure an almost immediate hyperplastic reaction.[5, 6, 7] Stimulation of erythropoiesis likewise protects the marrow from the destruction regularly produced in rabbits by the chemical agent, methyl-*bis*-(β-chloroethyl) amine hydrochloride. The degree of destruction of the marrow which regularly follows the administration of a single intravenous dose of 3 mg. of HN_2 or 800 r whole body x-irradiation is comparable from the histologic point of view through the fourth day after exposure to either of these agents. A significant anemia follows the latter (800 r) which reaches its maximum on the fourteenth day after exposure; whereas an anemia of only borderline significance is apparent after the former (HN_2). As is evident in the data presented, the histologic recovery of the marrow from intoxication with HN_2 only is so rapid that the brief but complete cessation of erythropoiesis is essentially masked.

The further fall of the erythrocyte values and hemoglobin, after HN_2 injection in animals already made anemic with phenylhydrazine (Group 4), is very likely due to continued hemolysis as a result of the combination of phenylhydrazine and HN_2. No evidence of significant hemolysis was apparent during this initial period in animals which received HN_2 only (Group 2), nor did further significant anemia develop in animals which had received only phenylhydrazine (Group 3). In previous experiments in which phenylhydrazine administration was followed by 800 r whole body x-irradiation, no further anemia developed immediately following x-ray (one to five days). It is therefore suggested that phenylhydrazine plus HN_2 exerts a synergestic hemolytic effect on circulating erythrocytes. After reaching a minimum of less than 2.8×10^6 per cubic millimeter, the erythrocytes of the animals which had phenylhydrazine and HN_2 rose so rapidly that a normal value was evident as rapidly as in the animals which received phenylhydrazine only (Group 3) and before the value of the group which received HN_2 only had fallen to its lowest point. The histologic findings indicate that a sufficient number of erythroblasts and hemocytoblasts survive the insult of the HN_2 in the latter group to immediately carry on normal erythropoietic activity and subsequently proceed to an intense hyperplasia and rapid recovery. In the group which received HN_2 only (Group 2), on the other hand, erythropoietic tissue was essentially destroyed and compensatory hyperplasia and recovery were comparatively slow. In previous reports[1, 2, 3] it was pointed out that whereas mitotic activity stopped in the bone marrow of normal rabbits during the first twenty-four hours after 800 r whole

body x-irradiation, mitotic activity in the marrow of animals with an erythroblastic hyperplasia continued at a normal or greater than normal rate after this dose of x-irradiation. The findings in the present study with HN_2 are identical in this respect with the x-ray studies. The free cells of the hematopoietic marrow which survive the insult of HN_2 are largely hemocytoblasts and basophilic erythroblasts. This fact is good evidence for support of the concept that the less mature cells are more resistant than more mature cells of the erythroblast series. If the more mature forms were less sensitive or of equal sensitivity to their precursors, one would expect to find a proportionate number of various maturation phases surviving at the early intervals after HN_2 particularly. In other words, one would expect surviving erythroblasts in each maturation phase to be in a proportion or ratio to one another more nearly like that of the hyperplastic marrow at the time of HN_2 administration if the sensitivity were equal. If the sensitivity of various maturation phases differs, then a predominance of the more resistant forms would be found in the early intervals after exposure to the noxious agent. Since the surviving free cells were predominantly basophilic erythroblasts and hemocytoblasts, one must conclude that under the conditions of this experiment these immature cells are less sensitive to HN_2 than the more mature forms in the marrow. It is further apparent that reticular cells not only retain their function of transformation to the free precursors of the marrow but also that the stimulation provided by the phenylhydrazine administration has probably increased this transformation to an extent which is significant in the development of the rapid hyperplasia and recovery.

Of interest is the fact that after HN_2 only (Group 2), granulocytopoietic, megakaryocytopoietic, and erythrocytopoietic tissue in the marrow is more completely destroyed than lymphatic tissue in the spleen, lymph node, and thymus. Recovery of the bone marrow and return of the circulating granulocytes, erythrocytes, and platelets to normal values, however, precede the complete histologic recovery of lymphatic tissue and the return of lymphocyte values of the peripheral blood to normal.

It is of even greater interest that recovery of the bone marrow from the insult of HN_2 (3 mg. per kilogram) is more rapid than the recovery from x-ray (800 r) even though the degree of destruction and atrophy is entirely comparable during the early phases (one to four days). If one assumes that this difference in recovery rate is on the basis of the fact that HN_2 has a more selective action than total body x-irradiation, then one is assuming that irradiation can produce an indirect suppression of marrow hematopoiesis or that irradiation produces greater effects on reticular cells which retard heteroplastic blood formation than does HN_2. The latter possibility seems more likely.

Significant regeneration of granulocytopoiesis and megakaryocytopoiesis in the marrow of animals which received phenylhydrazine and HN_2 (Group 4) occurred at a distinctly earlier stage than in the animals which received HN_2 only (Group 2). This regeneration occurs homoplastically from a few surviving myelocytes and heteroplastically from reticular cells and hemocytoblasts. This

histologic evidence of an earlier regeneration of granulocytes in the animals which received both phenylhydrazine and HN_2 (Group 4) correlates well with the studies on the peripheral blood as illustrated in Fig. 5.

The degree of destruction and time required for recovery of lymphatic tissue after the administration of HN_2 only (Group 2) appear to be entirely comparable with the destruction and recovery of lymphatic tissue after phenylhydrazine and HN_2 (Group 4).

These observations suggest that the production of a hyperplastic marrow prior to the use of x-irradiation or HN_2 might be useful as a means of protecting the normal hematopoietic tissue when these agents are used in therapy of such neoplastic disorders as the lymphomas. The fact that the persistent hyperplasia after HN_2 or x-ray is dependent in part on heteroplastic formation of the free marrow precursors from reticular cells suggests that venesection almost immediately before HN_2 or x-ray administration might effect a more rapid regeneration of the marrow from the destructive action of these agents. Phenylhydrazine administration would not seem particularly desirable under these conditions, but perhaps adequate and controlled stimulation could be achieved by exposure to a low atmosphere of oxygen or venesection.

SUMMARY

The effect of intravenous administration of a nitrogen mustard (methyl-*bis*-(β-chloroethyl) amine hydrochloride) in a dose of 3 mg. per kilogram was studied in normal rabbits and in rabbits with an anemia and erythroblastic hyperplasia produced by phenylhydrazine hydrochloride. Controls consisted of normal untreated rabbits and rabbits given phenylhydrazine alone. Histopathologic studies were made of animals from each group which were sacrificed at frequent intervals. Hematologic observations likewise were made at frequent intervals.

A single intravenous injection of HN_2 produced only a modest anemia of questionable significance in normal rabbits which was maximal at approximately three weeks. Leucopenia and a marked reduction in the number of circulating reticulocytes also were produced. A rapid destruction of hematopoietic tissue in the spleen, thymus, lymph nodes, and bone marrow occurred within twenty-four hours. The virtual absence of mitoses during the first twenty-four hours after the injection of HN_2 was striking; in stages up to the fifth day mitoses were seen only rarely. During the second, third, and fourth days after HN_2 the bone marrow was practically devoid of cells. Only a few scattered hemocytoblasts, erythroblasts, plasma cells, and giant myelocytes were seen. Erythrocytopoietic, granulopoietic, and megakaryocytopoietic recovery in the group occurred more or less simultaneously. Regeneration, evidence of which was first apparent on the fifth day, progressed to an essentially normal marrow by the tenth day. Lymphatic tissue though severely damaged by HN_2 was less atrophic than the bone marrow, but regeneration was slower and reconstruction was not apparent until after the fourteenth day. The appearance of leucopenia and reticulocytopenia correlated closely with the hematopoietic destruction, atrophy, and eventual reconstitution. The anemia which developed was modest, probably because of the rapid reconstitution of erythrocytopoiesis in the marrow.

Phenylhydrazine alone in a dose totaling 40 mg. produced an approximate 50 per cent reduction in erythrocyte and hemoglobin values with a simultaneous reticulocytosis and erythroblastic hyperplasia in the bone marrow. These hematologic values of the peripheral blood returned to normal by the twelfth day after phenylhydrazine withdrawal, but increased erythrocytopoiesis in the marrow persisted for about two weeks.

Animals to which 3 mg. per kilogram of HN_2 were given after an anemia and an erythroblastic hyperplasia of the bone marrow had been produced by phenylhydrazine developed further anemia within the first five days after the HN_2 administration, but recovery was complete by the twelfth day. *In fact, the hemoglobin and erythrocyte values of this group returned to normal as quickly as in animals which were given phenylhydrazine only.* The reticulocyte value of the animals which received phenylhydrazine prior to the HN_2 fell from a value of circa 25 per cent to 2 per cent by the third day after HN_2 but rose rapidly to 15 per cent by the sixth day and slowly fell to normal by about the fourteenth day. The marrow of these animals was extremely hyperplastic at the time at which the HN_2 was given. The usual evidence of destruction followed the HN_2 administration, but large numbers of cells in the bone marrow survived as compared with animals which were given HN_2 only. *The surviving cells were predominantly basophilic erythroblasts and hemocytoblasts and were present in an appreciably larger number than normally present in controls. Heteroplastic regeneration from reticular cells was conspicuous throughout the first six days after HN_2 administration.* Mitoses were present in large numbers even in the early stages after the HN_2 administration and increased rapidly to a maximum by about the fourth day. The more mature forms of the erythroblast series were progressively increased in the second, third, fourth, fifth, and sixth day stages after HN_2. A significant recovery of granulopoiesis and megakaryocytopoiesis in the marrow was apparent beginning on the fourth and fifth day after HN_2 in these animals. Hyperplasia in the marrow of these animals continued for about two weeks.

The fact that further anemia followed immediately after HN_2 administration in the group of animals which received phenylhydrazine and HN_2 cannot be interpreted as related to bone marrow destruction since a normal or greater than normal amount of erythrocytopoiesis was present during this period as evidenced by the histologic studies and the increased number of reticulocytes in the peripheral blood. It seems likely that this was a hemolytic effect due to a synergistic action of phenylhydrazine and HN_2 under the conditions of this experiment.

The initial effects of 800 r whole body x-irradiation on the hematopoietic system of normal animals and animals with a regenerative anemia and hyperplastic bone marrow are largely comparable with the initial effects produced by HN_2 insofar as the destruction of the bone marrow is concerned. The latter agent (HN_2), however, apparently intensifies the hemolytic action of phenylhydrazine on circulating erythrocytes; whereas x-ray does not. Regeneration of the bone marrow after HN_2 alone or phenylhydrazine plus HN_2 is more rapid than after x-ray alone or after phenylhydrazine plus x-ray.

It is suggested that stimulation of the hematopoietic system of the human being with disorders such as lymphomata might be feasible as a means of partial protection of or hastening the recovery of normal hematopoiesis from such destructive therapeutic agents as x-ray or HN_2.

CONCLUSIONS

1. In normal rabbits the intravenous injection of nitrogen mustard (methyl-*bis*-(β-chloroethyl) amine hydrochloride) in a dose of 3 mg. per kilogram body weight produces a modest anemia and a severe leucopenia and reticulocytopenia.

2. Nitrogen mustard in a dose of 3 mg. per kilogram when given to animals in which an anemia, reticulocytosis, and hyperplastic marrow are already present by virtue of prior phenylhydrazine hydrochloride administration produces:

(a) Further anemia within the first five days after the HN_2 administration which is presumed to be due to an apparent synergistic hemolytic action of phenylhydrazine and HN_2 on the circulating erythrocytes. No latent anemia is produced under these circumstances such as is produced by HN_2 alone. Recovery from the anemia induced by phenylhydrazine and HN_2 is as rapid as from the anemia produced by phenylhydrazine alone and faster than the recovery from the modest anemia produced by HN_2 alone.

(b) A leucopenia comparable with that produced by HN_2 only. Recovery from the leucopenia induced by phenylhydrazine and HN_2 is more rapid than the recovery after HN_2 only.

(c) A reduction of the reticulocyte value from circa 25 per cent to the normal value by the third day followed by immediate recurrence of the reticulocytosis. HN_2 alone, on the other hand, produces a marked reduction below normal values with a gradual return to a normal value.

3. In the normal rabbit HN_2 alone produces a marked depletion of lymphatic tissue, recovery from which requires more than two weeks. Bone marrow becomes extremely atrophic but regeneration begins by the fifth day. Mitotic activity which is virtually absent in these tissues for the first twenty-four to forty-eight hours after HN_2 administration is only gradually resumed.

In rabbits with an anemia, reticulocytosis, and hyperplastic marrow induced by phenylhydrazine hydrochloride, the intravenous injection of 3 mg. of nitrogen mustard per kilogram of body weight produces the usual evidence of destructive effects in the hematopoietic tissue, but large numbers of hemocytoblasts and erythroblasts in the bone marrow especially survive which are immediately capable of proliferation. Mitotic activity continues at a normal or greater than normal rate under these conditions.

4. The production of a hyperplastic (erythroblastic) marrow protects the marrow from the destructive effects of HN_2 by virtue of increasing the population of hemocytoblasts and basophilic erythroblasts which are less sensitive to this chemical agent than the more mature derivatives of these cells. Heteroplastic regeneration from reticular cells likewise probably plays a significant role in the rapidity of recovery.

5. More immature forms of the erythroblast series and hemocytoblasts are less sensitive to either a physical agent (x-ray) or a chemical agent (HN_2) than are the more mature forms of the same maturation series.

6. Prior stimulation of hematopoiesis in the human being as a means of partially protecting the hematopoietic tissue from such therapeutic agents as nitrogen mustard or irradiation may be feasible.

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DIETARY AND HORMONAL INFLUENCES IN EXPERIMENTAL UREMIA

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THE mechanism of uremia has not been defined in precise chemical terms. Still, on general grounds, treatment is directed toward control of azotemia and hyperpotassemia. The most obvious means to these ends are dietary and consist in restriction of exogenous protein and potassium and in the administration of high caloric diets consisting of carbohydrate and fat. Such diets slow the rate of endogenous protein catabolism and also inhibit the release of potassium from the cell stores. The advantages of such a regime, supplemented with blood transfusion and by antibiotics, has been demonstrated recently by Borst.[1]

In addition, theoretical and experimental considerations suggest that advantage might be taken of the actions of certain hormones. Thus, desoxycorticosterone acetate (DCA) prolongs life when given to animals prior to bilateral nephrectomy,[2, 3, 4, 5] and an antiuremic property, probably dependent on increased protein anabolism,[6] has been ascribed to testosterone.[7] Lastly, insulin, since it decreases potassemia and tends to slow the conversion of protein to glucose, might be of some value.[8]

While the general considerations underlying the treatment of uremia by high-caloric, nonprotein diets are entirely clear and valid, a survey of the field indicated a dearth of critically controlled experimental comparisons in significantly large groups of animals. The present study was therefore designed for the evaluation of the comparative effects of isocaloric diets of protein, fat, and carbohydrate on the survival time of rats subjected to bilateral nephrectomy and of the effect of hormones under controlled conditions of diet and fluid intake.

The first series of observations were made in normal animals and the second in animals in which infection was simulated by formation of turpentine abscesses. The distinction is important, since infection, with its acceleration of protein catabolism and discharge of protein metabolites and potassium into the blood, is a frequent complication in uremic patients.

SERIES I. NORMAL ANIMALS, BILATERALLY NEPHRECTOMIZED: EFFECTS OF DIET AND HORMONES

(a) Method.—The animals used were male albino rats, weighing 200 to 350 grams. The experimental groups were selected for equal weights in each because age is a determinant of survival after nephrectomy.[9, 10] Nephrectomy was done in two stages at intervals of

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seven days. Treatment was begun immediately after removal of the second kidney. Effects of each of the diets and of concurrent hormonal treatments were tested separately and, in each of the comparative series, a reference group given either protein or carbohydrate was used. The carbohydrate and fat diets were prepared in the manner of Reinecke, Ball, and Samuels[11] but without salt mixture and vitamins; the protein diet used was isocaloric and consisted of egg albumin, casein, and a protein digest. The diets were given twice daily by gavage in a daily volume of 3 c.c. per 100 sq. cm. of body surface. In each of the diets, this volume contained 10 calories. Body surface was calculated from the formula $S = KW^{0.66}$ where S is body surface in square centimeters, K a constant of 9.1, and W the weight in grams. The potassium contents of the diets were measured by the method of Shohl and Bennett[12] and found to be 1.1, 0.29, and 0.84 mg. per cubic centimeter respectively for protein, fat, and carbohydrate rations.

Steroid hormones were given subcutaneously in daily doses of 10 mg. of 0.4 c.c. hydroalcoholic suspension. Blood samples of 0.2 c.c. were taken from the tail at thirty and forty-eight hours after nephrectomy for determination of urea nitrogen by the method of Archibald.[13]

(b) Results.—The effects of treatment on survival time and on the level of blood urea nitrogen are summarized in Table I.

Inspection of the data shows first that survival decreases as azotemia increases. Second, comparison of Groups 1 (fasting) and 2 (protein-fed) shows that fasting, contrary to some impressions, is as deleterious as protein feeding. Third, comparisons of Groups 3 and 7, 8 and 12, and 13 and 17 indicate that survival is longer and azotemia less in carbohydrate- and fat-fed than in protein-fed animals. Last, survival is somewhat shorter and azotemia greater in animals given fat instead of carbohydrate, a difference which can be attributed to the diarrhea caused by a pure fat diet.

Treatment with hormones, singly or in combination, had no significant effect on survival and azotemia in animals fed either protein or fat. However, in animals fed only carbohydrate, blood urea nitrogen levels at thirty hours after nephrectomy were somewhat lower during treatment with DCA (Group 9) and testosterone (Group 10) than in the controls. The difference was even greater in animals given combined treatment of the two steroids (Group 11). Mean survival time was somewhat prolonged by hormonal treatment. To confirm this observation, the experiments were repeated on four groups (8a, 9a, 10a, and 11a) of ten animals each, which were treated exactly as were animals in Groups 8, 9, 10, and 11. In these groups blood urea nitrogen concentrations at thirty hours after nephrectomy were as follows: group 8a, 129 (range, 94 to 150) mg. per 100 c.c.; group 9a, 92 (71 to 100); group 10a, 112 (78 to 150); and group 11a, 70 (57 to 87). Thus, these observations establish the facts that testosterone and, curiously, DCA both tend to suppress azotemia in carbohydrate-fed, nephrectomized animals.

SERIES II. ANIMALS WITH SIMULATED INFECTION (TURPENTINE ABSCESS)

The purpose of this series of experiments was to test the effects of carbohydrate feeding as such and of the same diet when combined with steroid hormones and insulin under conditions of catabolic stress.

TABLE I. MEAN SURVIVAL TIME AND BLOOD UREA NITROGEN LEVEL IN BILATERALLY NEPHRECTOMIZED RATS

GROUP	DIET	TREATMENT	NUMBER OF RATS	SURVIVAL TIME (HR.)	BLOOD UREA NITROGEN (MG. %)	
					AT 30 HR.	AT 48 HR.
1	--	--	17	58 (47- 64)	227 (200-275)	388 (337-430)
2	Protein	--	10	60 (44- 82)	154 (140-192)	300 (245-325)
3	Protein	--	20	40 (36- 68)	250 (245-280)	540 (512-575)
4	Protein	DCA	10	44 (34- 57)	263 (216-297)	543 (460-605)
5	Protein	Testosterone	10	44 (37- 57)	240 (194-280)	461 (400-512)
6	Protein	DCA and testosterone	10	52 (38- 60)	281 (255-312)	510 (460-562)
7	Carbohydrate	--	10	86 (60-108)	167 (140-185)	237 (205-262)
8	Carbohydrate	--	20	93 (59-128)	112 (74-152)	134 (89-157)
9	Carbohydrate	DCA	9	127 (83-179)	89 (69-105)	131 (105-146)
10	Carbohydrate	Testosterone	9	118 (60-156)	100 (73-152)	130 (95-173)
11	Carbohydrate	DCA and testosterone	9	116 (70-156)	60 (46- 88)	90 (67-115)
12	Protein	--	9	53 (44- 68)	215 (162-282)	--
13	Fat	--	20	77 (52- 98)	175 (150-205)	298 (278-322)
14	Fat	DCA	18	76 (50-100)	167 (112-228)	220 (172-282)
15	Fat	Testosterone	18	74 (57-103)	173 (158-193)	298 (282-343)
16	Fat	DCA and testosterone	18	75 (39-100)	166 (147-191)	247 (238-255)
17	Protein	--	10	50 (41- 53)	239 (167-285)	455 (438-468)

(a) Method.—Thirty rats were divided into five groups. Following a two-stage nephrectomy, all the animals were fed a high carbohydrate diet by stomach tube. Supplementary treatment was started as indicated in Table II and turpentine was injected once subcutaneously into the prepectoral region in a dose of 0.5 c.c. in four of the groups. Protamine zinc insulin was given in a dose of 1 unit daily in two groups and the steroid hormones were given in doses of 5 mg. twice a day in another two groups. Blood urea nitrogen was determined thirty and forty-eight hours after nephrectomy. At forty-eight hours the animals were anesthetized and blood was collected for potassium determination.[18] Data are collected in Table II.

TABLE II. INFLUENCE OF INSULIN AND STEROID HORMONES ON THE MORTALITY AND BLOOD UREA NITROGEN OF NEPHRECTOMIZED RATS INJECTED WITH TURPENTINE AND FED CARBOHYDRATE

TREATMENT	BLOOD UREA NITROGEN (MG. PER 100 C.C.)		SERUM POTASSIUM (MG. PER 100 C.C.)	NUMBER OF DEATHS AT 48 HR.
	AT 30 HR.	AT 48 HR.		
--	111 (74-142)	156 (125-186)	35	0
Turpentine	156 (127-172)	242 (202-275)	45	1
Turpentine and insulin	182 (157-204)	241 (210-280)	42	1
Turpentine and insulin and DCA and testosterone	179 (167-215)	252 (217-277)	43	3
Turpentine and DCA and testosterone	174 (147-202)	238 (210-272)	46	1

(b) Results.—The increased azotemia and mortality at forty-eight hours consequent on turpentine abscesses were not influenced by hormonal treatment. Furthermore, animals which were not given turpentine (group 1) remained in good condition (temperature, muscular tone, reactivity), while those with turpentine abscesses were apathetic, asthenic, cold, and had buccal hemorrhages. Morbidity and mortality corresponded roughly with the serum potassium contents. Therefore, under the present experimental conditions, neither steroid hormones nor insulin proved to be of any advantage over feeding a high carbohydrate, high caloric diet alone, and the diet did not prevent the deleterious effects of simulated infection.

DISCUSSION

In earlier studies, Lyon[15] and Lyon, Shafton, and Ivy[16] showed that the mean survival time of nephrectomized dogs fed on bread, milk, and lactose was 137.6 hours as compared with 105.6 hours for dogs fed on a meat diet. In contrast, Winternitz, Mylon, Waters, and Katzenstein[17] reported no beneficial effects of a carbohydrate feeding to nephrectomized dogs, taking as criteria of benefit blood urea and survival time. Their animals, however, were treated with fluids and alkalis, in order to maintain normal blood volume and alkaline reserve. There is no proof that such procedures are of real benefit, while it is well recognized that anuric animals do not withstand excess fluid or electrolytes. Such treatment might therefore obscure any effect of diet. The harmful effects of water have been demonstrated by Bergman and Drury[9] and by Birnie, Eversole, and Gaunt.[10] Bergman and Drury[9] also showed that preoperative fasting does not influence significantly the survival time, and that meat is definitely toxic while glucose improves the condition of the rats.

They obtained a definite prolongation of survival time by daily administration of as little as 5 c.c. of a 20 per cent glucose solution. Since this represents only 4.5 calories, in contrast to the average daily intake of about 50 calories given in our experiments, it seems carbohydrate is beneficial even though it does not meet the caloric requirements. Mylon and Goldstein[18] found also that preoperative feeding of a carbohydrate diet to dogs subjected to nephrectomy or renal artery ligation prolongs the survival time and delays the blood nonprotein nitrogen rise which occurs with preoperative meat feeding. The toxic effects of protein in experimental uremia produced by ligation of the vena cava above the renal veins have also been demonstrated by Addis and Lew.[19] More recently Addis[20] showed that protein diet has a deleterious influence in animals subjected to sudden loss of three-fourths of the renal tissue.

These results and our own confirm the well-established principles that high caloric carbohydrate and fat diets have an inhibitory effect on protein catabolism which in turn delays or diminishes uremia.

DCA given preoperatively has been shown to prolong the survival time of nephrectomized rats and mice.[2, 3, 4] However, Winkler, Smith, and Hoff[21] were unable to demonstrate any beneficial effect from DCA or adrenocortical extract given postoperatively to dogs made anuric by ureteral ligation. They suggested that the probable protective mechanism of DCA pretreatment lies in its depletion of the body stores of potassium. That this may be so is shown by the increased survival of nephrectomized animals preoperatively depleted of potassium by diet alone.[22, 23] Rodbard[5] has confirmed the fact that the life of nephrectomized dogs can be prolonged by preoperative treatment with DCA. He also observed some beneficial effect from DCA given postoperatively which was unrelated to the level of serum potassium. Our data also indicate a slight effect on survival and a more definite decrease in azotemia from postoperative treatment with DCA and testosterone in rats fed only on carbohydrate and thus confirm Selye and Nielsen[24] as concerns DCA. Selye[6] has also observed a beneficial effect of testosterone given pre and postoperatively to nephrectomized mice. Since the effect cannot be ascribed to renal potassium depletion,[25] it must depend on the protein anabolic property of the steroid.

Our data emphasize the importance of diet in experiments on anuric animals. They also suggest that some of the contradictory results of postoperative treatment with hormones as to survival time and blood nonprotein nitrogen can be attributed to differences in the diets on which the different groups of animals were maintained in the several laboratories. While the dietary data form a basis for clinical treatment, the hormonal effects obtained during treatment with massive doses can hardly serve as recommendations for a clinical trial.

Finally, although high carbohydrate and fat diets can prolong survival and diminish the rate of increase of azotemia in nephrectomized animals, they are ineffective in countering the protein catabolic impulse caused by turpentine abscess. This is a confirmation of Borst's observation[1] that the effective-

ness of dietary treatment depends on the control of infection. These and other aspects of the treatment of uremia have been reviewed editorially[26] and the recommendation has been made that Borst's protien-free regime has more theoretical merit than the low protein ration of Black and Stanbury.[27]

SUMMARY

Effects of fasting and various high isocaloric diets consisting of carbohydrate, fat, or protein were tested in bilaterally nephrectomized rats.

Fasting is not more advantageous than protein feeding. Carbohydrate and fat are definitely more beneficial in terms of survival and azotemia than protein diet, and, of the two, carbohydrate feeding is better tolerated.

Desoxycorticosterone acetate and testosterone alone or in combination were somewhat effective only in rats fed a carbohydrate diet; the most significant change was a decrease of azotemia following combined treatment with both steroids.

High carbohydrate diet alone or in combination with DCA, testosterone, or insulin did not influence the course of uremia in nephrectomized rats subjected to turpentine abscess.

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EFFECT OF AN ACID AND ALKALINE SALT ON THE URINARY EXCRETION OF IRON

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MUCH work has been done on the absorption, utilization, and excretion of iron, and although new information is accumulating there is still confusion in respect to many of the metabolic processes concerned. The amount of iron ordinarily excreted in the urine is small,[1, 2, 3] although a study on 100 men and 100 women[4] showed a greater excretion than had many of the previous investigations and also revealed a higher urinary iron excretion in women than in men, 0.489 mg. per day as compared with 0.395 milligram. The urinary iron excretion was found to be fairly constant in the same individual from day to day. There is a marked and prompt increase in the urinary iron excretion following intravenuous administration of iron[5, 6] although no appreciable increase occurs in the iron content of the feces under these circumstances. Little, Power, and Wakefield[5] thought that this was due to the marked and rapid increase in the serum iron concentration, and that it probably did not occur with normal iron absorption from the intestine. Our previous investigation[4] did not show an increase in the urinary iron excretion when large amounts of iron were given by mouth or with intramuscular iron injections given to the point of tolerance. Although previous extensive studies have failed to clarify all phases of iron metabolism, it appears that iron is absorbed more readily when in a ferrous state than as a ferric salt, that an acid reaction in the stomach favors the absorption of dietary iron, and that absorption is regulated by the body need.[7, 8, 9]

The present study was undertaken in order to ascertain the effects of an acid and an alkaline salt on the urinary excretion of iron. It has been demonstrated that an acid media in the stomach favors absorption of dietary iron, but further effects of alkalinization, such as is commonly employed in the treatment of peptic ulcer, on iron metabolism have not been studied adequately.

PROCEDURE

This study was carried out on twenty-one persons (11 men and 10 women). Ten of the subjects had peptic ulcers. The remainder were patients on the medical wards who had no disease which would interfere with intestinal absorption and who were not passing blood from the urinary or gastrointestinal tract. The subjects continued with their ordinary routine and with their hospital diets—the iron contents of which were calculated from the table of Waller.[10] No patients were receiving or had received iron medication so that no abnormal or sudden increase in the serum iron was induced. No medications were given aside from the alkaline or acid salts. Fluids were allowed as desired at all times.

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During the control period, twenty-four-hour urine specimens were collected for three days in glass containers which were especially cleaned to prevent iron contamination. Creatinine determinations were done each day to check the completeness of collection. Iron determinations were done in triplicate on aliquots of the pooled three-day specimens using the method of Reis and Chakmakjian.[11]

Following a control period of three days, the patient was given 1 Gm. of sodium bicarbonate six times a day, and twenty-four hour urine specimens were collected for a three-day period and handled in the same manner as in the control period. After an interval of one day, during which the specimens were discarded, eleven of the subjects received 1 Gm. of ammonium chloride four times a day for a three-day period. Specimens were handled as before.

Hemoglobin determinations were made at the beginning of each three-day period by the acid hematin method using a photoelectric colorimeter. A hematocrit was done at the same time by the Van Allen method.[12] All values were well within the range of normal, and the periods of observation were of too short duration to note any significant changes in hemoglobin or hematocrit values.

RESULTS

A summary of the significant findings is presented in Table I in which the data for the male and female patients are presented separately.

TABLE I. EFFECT OF AN ACID AND ALKALINE SALT ON THE URINARY EXCRETION OF IRON*

				URINE SUMMARY (AVERAGE PER DAY)								
		BLOOD		CONTROL			SODA BICARBONATE			AMMONIUM CHLORIDE		
PATIENT	AGE	HEMOGLOBIN (GM.)	HEMATOCRIT (%)	VOL. (C.C.)	SP. GR.	Fe (MG.)	VOL. (C.C.)	SP. GR.	Fe (MG.)	VOL. (C.C.)	SP. GR.	Fe (MG.)
				Men								
1	43	–	–	857	1.026	.15	863	1.024	.09			
2	50	11.78	40.5	2,073	1.012	.41	2,150	1.014	.22			
3	29	12.41	43.0	1,997	1.017	.60	3,553	1.012	.36			
4	47	13.47	44.0	1,743	1.013	.44	1,900	1.015	.24			
5	65	13.75	43.5	1,220	1.020	.31	1,303	1.019	.16			
6	52	10.73	40.0	867	1.021	.26	1,057	1.020	.16	1,017	1.019	.38
7	31	12.41	44.5	1,923	1.011	.38	1,607	1.013	.20	1,827	1.014	.46
8	28	12.62	42.0	650	1.034	.23	973	1.026	.19	873	1.027	.44
9	31	12.62	44.0	1,760	1.016	.44	1,683	1.017	.34	1,287	1.019	.45
10	43	13.75	46.0	1,827	1.012	.37	1,637	1.012	.25	2,240	1.010	.89
11	29	14.38	45.5	1,020	1.020	.29	1,280	1.017	.22	940	1.017	.31
Average						.35			.22			.49
				Women								
12	24	6.38	23.0	1,653	1.014	.33	1,463	1.016	.15			
13	42	10.20	37.0	1,527	1.008	.23	1,580	1.008	.16			
14	49	10.22	39.0	2,907	1.011	.44	2,643	1.011	.26			
15	39	10.94	38.0	1,897	1.013	.38	1,483	1.015	.16			
16	48	11.99	40.0	2,953	1.006	.59	3,047	1.007	.46			
17	41	10.37	38.0	1,320	1.011	.33	1,863	1.009	.15	2,380	1.011	.71
18	49	10.94	37.5	1,938	1.009	.58	1,970	1.009	.30	2,367	1.008	1.18
19	40	11.36	36.0	1,213	1.020	.49	1,613	1.014	.40	1,846	1.014	.74
20	26	11.36	41.0	720	1.019	.18	1,097	1.017	.11	1,400	1.013	.56
21	48	11.57	38.0	1,250	1.021	.25	703	1.023	.13	1,533	1.021	.38
Average						.38			.23			.71

*On subjecting these data to analysis, the results are found to be statistically significant. Applying the t-test to the relationship between the control period and the period in which soda bicarbonate was administered, we find that $t = 3.97$ with 40 degrees of freedom, which indicates a highly significant difference. Similarly, in comparing the difference between the control period and the period of ammonium chloride administration we find $t = 3.24$ with 30 degrees of freedom, again a statistically significant difference.

Administration of Sodium Bicarbonate.—It will be noted in the table that without exception, after the administration of this alkaline salt there was a drop in the daily urinary iron excretion varying from .04 mg. (Patient 8) to .28 mg. (Patient 18) per day, or from 15 per cent (Patient 8) to 54 per cent (Patient 12). Analysis of these figures revealed the changes to be statistically significant. The soda bicarbonate produced no consistent or significant change in the amount or specific gravity of the urine. The average daily urinary iron excretion for men during the control period was .35 mg. and during the period of alkalinization was .22 mg. or a decrease of 37 per cent. In the women the average excretion fell from .38 to .23 mg. per day or a decrease of 39 per cent.

Administration of Ammonium Chloride.—This medication was not given to those patients with peptic ulcer but was subsequently administered to the eleven other patients. The urinary iron excretion was increased in every case, not only above the period during which soda bicarbonate was given but above the control period as well. In two cases the increase was slight, being .01 and .02 mg. respectively, whereas in Patient 18 the increase was .60 milligram. Analysis again revealed these changes to be of statistical significance. In the men the average increase above the control level was 40 per cent, and in the women, 86 per cent. The period of ammonium chloride administration was associated with an increased urine volume in all female patients but in only three of the men.

DISCUSSION

These data show that the administration of an alkaline salt diminishes the amount of iron excreted in the urine, whereas the administration of an acid salt causes a marked increase when given to patients on a normal dietary iron intake. Previous work[13] has shown that achlorhydria decreases the retention of iron from a normal dietary iron intake, and Mettier and Minot[14] have shown that a better utilization is obtained from small amounts of iron when given in an acid medium. Lintzel[15] states that iron is absorbed only in an ionized form and that an acid reaction is necessary for this ionization. These data on the urinary iron excretion are in keeping with the foregoing observations on the absorption of iron from an acid media.

CONCLUSIONS

The urinary iron excretion from a normal dietary iron intake is increased when an acid salt is administered by mouth and decreased when an alkaline salt is given.

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A CONSIDERATION OF SOME FACTORS IN URINE WHICH CAUSE THE PRECIPITATION OF HEMOGLOBIN IN VITRO

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ACID urine has been considered deleterious to patients with intravascular hemolysis.[1] That this opinion is generally accepted is indicated by the fact that alkalinization of urine in such individuals is common practice. However, experimental evidence for the view that acidity of urine exerts any influence on the solubility of hemoglobin is inconclusive. Likewise, whether acid urine predisposes to subsequent hemoglobinuric nephrosis is controversial.[2, 3, 4, 5, 6] Baker and Dodds[1] were the first to suggest that concentrations of NaCl in excess of 1 per cent in acid urine would effect a precipitation of hemoglobin. To our knowledge, these studies have never been repeated. Nor, what would seem more interesting, has it been shown that urine, not modified by the addition of salts or acids, when mixed with a solution of hemoglobin is capable of effecting its precipitation.

While evaluating the effect of alterations that occur in urine during starvation and dehydration on the production of experimental hemoglobinuric nephrosis in rabbits, it was found that some specimens of urine, when mixed with a solution of hemoglobin, caused a precipitation. It, therefore, seemed desirable to establish whether a similar phenomenon existed in human urine. Several samples from patients were found which, after mixing with a solution of hemoglobin, caused a precipitation at room temperature. Since such a reaction might be of importance in the production of hemoglobinuric nephrosis, it was decided to repeat some of the experiments of Baker and Dodds in order to determine which factors in urine are responsible for the precipitation of hemoglobin.

METHOD

Urine samples were collected from patients or obtained from the diagnostic laboratory following routine urine analyses. The urine was filtered through Whatman No. 40 filter paper before any studies were done. The specific gravity was ascertained with a hydrometer and the pH was determined with a glass electrode. Five milliliters of filtered urine were pipetted into duplicate tubes and mixed with 0.5 ml. hemoglobin solution (7 to 9 Gm. per cent) which was prepared as described previously.[7] Following agitation the mixture was examined for visual turbidity or precipitation at ten-, twenty-, and forty-minute intervals. In view of the fact that the pH was in excess of 5.5 in most of the samples, which is insufficient to cause a precipitation of hemoglobin spontaneously, it was not possible to evaluate the effect of greater acidity on the solubility of hemoglobin in a significant number of samples. In order to test the influence of acidity, as well as to repeat some of the experiments of Baker

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and Dodds, glacial acetic acid was added to the urine hemoglobin mixtures in which turbidity or precipitation had not occurred in forty minutes. Sufficient glacial acetic acid was used to depress the pH from the original levels to a range such as might be encountered during acidosis, approximately a pH of 5. Depending on the combined buffering capacity of urine and the hemoglobin, 1 to 2 drops (0.05 to 0.1 ml.) were necessary for each tube. Lowering the pH to 4.8 to 5.2 with acetic acid caused turbidity and precipitation in approximately half of the urine samples. In order to observe the effect of chlorides on hemoglobin solubility in acid urine, as well as to determine whether other ions exert any influence, the following tests were carried out: (1) urine samples were analyzed for their ash content by Neumann's method[8]; (2) chlorides were determined by Whitehorn's modification[9]; (3) inorganic phosphorus was determined by the method of Youngburg[10]; (4) inorganic sulfate, by Folin's method[11]; (5) urinary ammonia was determined by Conway's microdiffusion technique.[12] These determinations were done on urine samples which caused the precipitation of hemoglobin without acidification, and on ninety-three samples following the addition of acid. In the latter group approximately 50 per cent of the urine samples showed precipitation. Such studies permitted an analysis of the relationship between the concentration of chloride, sulfate, and phosphate anions and the presence or absence of precipitation.

To test the effect of various electrolytes on the solubility of normal and modified hemoglobin, experiments were done with solutions consisting of mixtures of a given salt and hemoglobin at pH levels of 4.5 to 5.2. The influence of acidification of hemoglobin previous to mixing with the salts was determined by spectrophotometric absorption at different wave lengths in a Coleman spectrophotometer.

RESULTS

When urine renders hemoglobin or its derivatives insoluble, one may observe a number of physical changes. Red hemoglobin is converted to a denatured protein, brown in color. The soluble hemoglobin becomes turbid, either before or after the color change occurs. The turbidity may persist, or, in turn, be followed by the formation of visible aggregates which eventually precipitate. Some urine samples will precipitate minimal quantities, whereas others can effect a complete precipitation of hemoglobin derivatives. Since the quantity which precipitated was not measured, the results are expressed either as positive or negative. Human hemoglobin, when added to distilled water and acidified slowly, forms a few light-colored aggregates without any evidence of turbidity. A comparable reaction was observed rarely in urine and commonly in salt solutions. Hemoglobin in distilled water at a pH of approximately 5.0 was used as a control to ascertain if the test sample caused precipitation.

Following the filtration of urine and mixing with a solution of hemoglobin, seven of 302 samples caused precipitation at room temperature within forty minutes. Turbidity without precipitation, which we consider as evidence of minimal reaction, occurred four times. Therefore, eleven of 302 urine samples caused precipitation of hemoglobin derivatives. The pH values in these eleven samples varied from 5.1 to 5.8. In the remaining 291 samples the pH was higher than 5.5, over 90 per cent falling between 5.5 to 6.8.

During these studies it became apparent that temperature exerts a decided influence on the rapidity with which precipitation occurs. To evaluate the influence of temperature, sixty-six urine samples were tested simultaneously at room temperature (25 to 31° C.) and at 37.5° C. In fifty-nine of

sixty-six samples neither turbidity nor precipitation occurred. In this group the temperature did not exert any apparent influence. In the remaining seven urine samples precipitation occurred once; four revealed turbidity, whereas two were negative at room temperature after forty minutes. Samples of the same urine, when mixed with hemoglobin and placed in the incubator at 37.5° C., all caused precipitation within forty minutes.

The anion concentrations in seven urine samples which caused precipitation at 37.5° C. are shown in Table I.

TABLE I. ELECTROLYTE CONCENTRATIONS IN URINE WHICH PRECIPITATED HEMOGLOBIN WITHOUT ACIDIFICATION

		URINE							
PATIENT	DIAGNOSIS	SPECIFIC GRAVITY	pH	ASH (MG. %)	NH_3^+ (MEQ./L.)	Cl^- (MEQ./L.)	$SO_4^=$ (MEQ./L.)	$H_2PO_4^-$ (MEQ./L.)	TOTAL ANIONS (MEQ./L.)
P. R.	Chronic glomerulonephritis	1.022	5.3	1,154	5.7	41.4	45.0	32.3	118.7
A. J.	Bronchiectasis	1.029	5.2	1,374	5.8	41.2	74.8	37.7	153.7
D. L.	Arteriosclerosis	1.023	5.1	1,030	9.1	34.3	69.0	26.7	130.0
H. M.	Hypertension	1.020	5.2	1,014	5.3	38.1	47.2	20.3	105.6
K. E.	Myxedema	1.020	5.1	1,030	3.4	60.6	50.6	17.4	128.6
C. T.	Ruptured intervertebral disc	1.027	5.4	990	13.1	20.3	62.0	29.3	111.6
C. T.	Ruptured intervertebral disc	1.009	5.8	314	6.7	17.1	14.8	8.7	30.6

The anion and ash concentrations were high in six of seven samples which were analyzed. The pH values varied from 5.1 to 5.8; in six samples the pH was 5.4 or less. Why precipitation occurred in the last urine sample is not apparent, since neither the pH nor the anion concentration is comparable to that of the other six samples. The findings show that hemoglobin is rendered insoluble by urine and suggest that the pH, electrolyte concentration, and temperature exert an effect on hemoglobin solubility.

It seemed desirable at this point to establish whether any of us could excrete urine which was capable of precipitating hemoglobin in vitro. Water was restricted to help increase the urine specific gravity. Acid-producing diets, augmented by the oral ingestion of mandelic acid and sodium sulfate separately at spaced intervals, were also taken. It was difficult to depress the urine pH, and the thirst became oppressive. However, after four days on this regime two of five were successful in excreting a urine which did precipitate hemoglobin derivatives within forty minutes at 37.5° C.

Observations on the relationship of urine anion concentration and in vitro hemoglobin precipitation following acidification were made in ninety-three samples of urine. The anion concentrations are expressed in milliequivalents per liter of urine. Since the pH was lowered in each case by the addition of acetic acid to a range of 4.8 to 5.2, the ratio of $H_2PO_4^-$ to $HPO_4^=$ would be, as is diagrammatically illustrated by Gamble,[13] approximately 50 to 1. For this reason the phosphates are calculated in milliequivalents of $H_2PO_4^-$. The relationship of precipitation with respect to the various anions at varying concentrations is shown in separate columns in Table II. The range of total

measured anion concentration in single urine samples is also shown. The frequency of precipitation is expressed in fractions, e.g., 3/8 indicates that precipitation occurred in three of eight urine samples at the specified anion concentration.

TABLE II. RELATIONSHIP OF IN VITRO HEMOGLOBIN PRECIPITATION FOLLOWING ACIDIFICATION TO THE ANION CONCENTRATION

Cl^-*		$SO_4^=$		$H_2PO_4^-$		TOTAL ANIONS†	
MEQ./L.	PRECIPITATION	MEQ./L.	PRECIPITATION	MEQ./L.	PRECIPITATION	MEQ./L.	PRECIPITATION
0-20	5/13	0-20	2/38	0-10	7/36	0-25	0/1
21-40	2/14	21-40	16/26	11-20	15/33	26-50	1/6
41-60	5/19	41-60	17/21	21-30	15/18	51-75	0/18
61-80	7/14	61-80	5/5	31-40	4/4	76-100	4/13
81-100	9/15	81-100	3/3	41-50	2/2	101-125	4/17
101-120	7/9					126-150	12/13
121-140	6/7					151-175	14/15
141-60	1/1					176-200	8/8
161-180	1/1					201-225	2/2

*Data of the various anions in the first three columns is tabulated on the basis of concentration and not separate urine samples.

†The sum of Cl^-, $SO_4^=$, and $H_2PO_4^-$ in a given urine sample is shown in the last column.

The relationship of precipitation to individual anion concentration is shown in the first three columns. The chloride concentration was the greatest in most of the samples. Not evident from the table, however, is the fact that the separate anions fluctuated over a wide range independently of each other. This fact explains the presence of precipitation at some of the lowest Cl^-, $SO_4^=$, and $H_2PO_4^-$ concentrations. In such urine samples the concentration of the other two anions was usually elevated above average values. The presence of precipitation at the lowest anion concentration in all three columns indicated that this phenomenon is not due solely to the action of a single anion. As the milliequivalent concentration of the different anions increases respectively, the ratio of hemoglobin precipitation also rises.

Examination of the total anion concentration in single urine specimens shows a considerable variation. The range from 51 to 175 meq. per liter includes the majority of the urine samples. Precipitation occurred in nine of fifty-five samples in which the total anion concentration was 125 meq. per liter or less. In a majority of these which caused precipitation the chloride concentrations tended to be low, whereas the SO_4 values were above average. In thirty-eight samples with a total concentration of 126 meq. per liter or more, hemoglobin precipitation occurred in thirty-six samples. It is apparent that, when the total anion concentration exceeds 126 meq. per liter, hemoglobin precipitation almost always occurs at pH values close to 5.0.

A comparison of results in Tables I and II indicates the following: The total concentration of the anions measured in urine samples which caused precipitation without the addition of salts or acids is in agreement, with but one exception, with the values observed in urine samples following acidification. Interestingly, the $SO_4^=$ concentrations were higher in the unmodified samples. In some of the urine samples the hemoglobin solubility was not affected as anticipated. That is, precipitation occurred at unusually low total

anion concentrations or in the intermediate range (76 to 125 meq. per liter). The cause for hemoglobin precipitation in the unusually low concentrations is not evident. However, when precipitation was observed at concentrations of 76 to 125 meq. per liter it was customary to have low Cl^- and high $SO_4^=$ values.

To determine the influence of different electrolytes on precipitation in a pH range of 4.5 to 5.2, a series of experiments was performed with pure salt solutions and hemoglobin. Solutions of normal and modified hemoglobin were used. The hemoglobin was changed by direct acidification with N/10 HCl until the pH was depressed to 4.92. This acidified hemoglobin was then incubated at 37.5° C. for sixty minutes. Prior to mixing with pure salt solutions, the normal and acidified hemoglobin were diluted 1:200 in 0.1 per cent Na_2CO_3 and analyzed spectrophotometrically. The absorption curve of the acidified hemoglobin was slightly modified; it crossed the curve of normal hemoglobin at 585 and 512 mμ, indicating that acidification and incubation had caused a change in the hemoglobin molecule. Solutions of pure salts at different molar concentrations in 5 ml. quantities were mixed with 0.5 ml. of hemoglobin, and the pH was adjusted to approximately 5.0, when necessary, with N/10 HCl. The results of some of the studies with mixtures of pure salts and solutions of normal and partially denatured hemoglobin are shown in Table III.

TABLE III. THE INFLUENCE OF DIFFERENT SALT CONCENTRATIONS AND THE PURITY OF HEMOGLOBIN ON PRECIPITATION IN THE SPECIFIED pH RANGES

SALT		NORMAL HEMOGLOBIN				ACIDIFIED HEMOGLOBIN			
NaCl	Molar concentration	0.5	0.4	0.3	0.2	0.4	0.3	0.2	0.1
	pH	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.1
	Reaction	–	–	–	–	+	+	–	–
$(NH_4)_2SO_4$	Molar concentration	0.4	0.36	0.32	0.28	0.2	0.1	0.05	0.025
	pH	5.4	5.0	5.0	5.4	5.2	5.2	5.3	4.9
	Reaction	–	–	–	–	+	+	–	–
Na_2SO_4	Molar concentration	0.5	0.4	0.3	0.2	0.2	0.1	0.05	0.025
	pH	5.1	5.1	5.2	5.1	5.3	5.2	5.2	5.2
	Reaction	–	–	–	–	+	+	+	–
Na_2HPO_4	Molar concentration	0.1	0.05	0.038	0.025	0.2	0.1	0.05	0.025
	pH	5.0	4.9	5.1	5.1	5.1	5.2	5.1	4.9
	Reaction	+	+	–	–	+	+	–	–
NaH_2PO_4	Molar concentration	0.5	0.4	0.3	0.2	0.5	0.45	0.4	0.35
	pH	4.7	4.8	4.9	5.0	4.5	4.6	4.6	4.6
	Reaction	–	–	–	–	+	+	–	–

Reactions followed for twenty minutes. Hemoglobin acidified and the pH of salt mixtures adjusted by N/10 HCl acid.

When normal hemoglobin was mixed with solutions of NaCl, $(NH_4)_2SO_4$, Na_2SO_4, and NaH_2PO_4 in which the molar concentrations varied from 0.5 to 0.1, precipitation did not occur in twenty minutes. Addition of hemoglobin to Na_2HPO_4 solutions in molar concentrations of 0.1 to 0.05 produced minimal precipitation following the adjustment of pH to 4.9 to 5.1. The difference observed when using Na_2HPO_4 instead of NaH_2PO_4 solutions is probably related to the initial high pH value of Na_2HPO_4, since the final ratio of $\frac{H_2PO_4^-}{HPO_4^=}$

would be equal after the pH was adjusted. The pH values for NaH_2PO_4 are lower than for the rest of the salts because of the difficulty encountered in adjusting the pH to 5.0. The partly modified hemoglobin, on the other hand, was almost completely precipitated within twenty minutes by the higher concentrations of all the electrolytes used.

DISCUSSION

Proteins have been shown to be least soluble in the neighborhood of their isoelectric points.[14] In this study hemoglobin was not precipitated by urine at or near its isoelectric point of 6.4. It is felt, therefore, that the in vitro influence of urine on hemoglobin solubility is not due to this factor. Hemoglobin is known to be amphoteric; it acts as an acid on the alkaline side of its isoelectric point and as a base below a pH of 6.4 when it has a net positive charge.[14] In view of this, one would expect the hemoglobin to bind the anions Cl^-, $SO_4^=$, and $H_2PO_4^-$ more strongly at a pH of 5.0 than at its isoelectric point. Furthermore, it is well known that ions of higher valence are more effective precipitating agents. In this respect the SO_4 anion would be quite effective since at a pH of 5.0 it exists almost entirely in the bivalent form.

Green has shown that electrolyte concentration exerts a decided influence on hemoglobin solubility at different pH levels.[15] Green's experiments, however, were conducted at pH levels higher than 5.8. Likewise, her studies were done with unmodified hemoglobin, whereas the present studies are complicated by changes in hemoglobin and the presence in urine of other solutes which might either augment or inhibit the effect of electrolytes. It is apparent that hemoglobin precipitation in urine in vitro is a complex phenomenon in which multiple variables are operating. At the present time, the in vitro hemoglobin precipitation can be attributed to pH and anion alterations. However, an explanation for the mechanism of precipitation will require further study and would appear to entail individual study of the variables known to exist in this system.

Baker and Dodds[1] originally suggested that two entirely independent factors are required for the precipitation of hemoglobin in urine. First, a pH of 5 to 6 is required to convert hemoglobin to methemoglobin and possibly acid hematin. Second, the urine must have a minimal concentration of about 1 per cent NaCl. Our studies are in agreement with the first suggestion, that acid urine which converts hemoglobin to other derivatives is conducive to precipitation. However, our findings do not support the hypothesis that a NaCl concentration in excess of 1 per cent is required. In our study, the urine samples which caused in vitro precipitation of hemoglobin never had a calculated NaCl concentration of 1 per cent. We did, however, find a close relationship between the total anion concentration and precipitation in urine samples in which the pH is 5.4 or less.

It became evident that three factors are necessary before urine causes precipitation of hemoglobin: an acid urine of proper pH, anions in sufficient concentration, and last, a modified hemoglobin. Initially we were not aware

that hemoglobin modification markedly accelerates precipitation until a study of the influence of pure salts on hemoglobin solubility was undertaken. Baker and Dodds do mention denaturation as a factor in conjunction with acid urine. However, one is not apt to consider hemoglobin changes of significant importance because a pH of 5.0 to 5.4 is desirable in urine before the ions begin to exert significant activity. In view of this, one is likely to assume that hemoglobin modification is merely an associated change rather than a factor which exerts a decided influence on the rapidity and quantity of precipitation.

Whether the ability of urine to precipitate derivatives of hemoglobin is of any importance in the production of hemoglobinuric nephrosis remains to be established. We are able to confirm that acidification of human urine within the physiologic range causes precipitation of modified hemoglobin. It was possible to show that unmodified human urine with the proper acidity and anion concentration also precipitates hemoglobin derivatives. It is hazardous to conclude from in vitro experiments whether precipitation will occur in vivo under similar conditions. However, one cannot help but wonder whether the mechanisms responsible for hemoglobin precipitation in vitro are not also in part responsible for intratubular hemoglobin cast formation.

CONCLUSIONS

It has been possible to confirm the observations that acidification of urine within the physiologic range will effect a precipitation of hemoglobin by some of the urine samples.

Urine which is not modified by the addition of either salts or acids and which is capable of precipitating modified hemoglobin can be obtained from some patients. Healthy individuals existing on an acid-producing diet which is augmented by the oral ingestion of mandelic acid and a limitation of water intake are able to excrete urine capable of effecting precipitation of hemoglobin.

Urine which precipitates hemoglobin in most instances would appear to possess several variables. In addition to pH and anion concentration studied in this work, it would seem that the known and unknown factors, which are responsible for modification of hemoglobin, play an important role in this system.

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THE USE OF HYPERTONIC SOLUTIONS FOR ENTERIC PERFUSION

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THE concept of extrarenal elimination of waste products from the blood stream across living membrane barriers is not new. For years diaphoresis and purgation of the gastrointestinal tract were commonly used as a means of eliminating toxic and nitrogenous products from the blood stream. The work of Pendleton and West,[20] Wells,[27] and others placed intestinal purgation on a more scientific basis by demonstrating that the mucosa of the intestine acted as a semi-permeable membrane permitting the movement of urea, sugar, and salts from the blood stream into the lumen of the bowel.

Enteric lavage utilizing the stomach, duodenum, jejunum, ileum, and colon has been effected in cases of azotemia, eliminating metabolic waste products by dialysis with varying degrees of success.[19]

Enteric perfusion offered attractive features of simplicity of clinical application and safety to the patient. Experimental evidence and reports of clinical application of intestinal perfusion[19] have clearly indicated that this method, though effective in lowering the nonprotein nitrogen of the circulating blood stream, has proved, for the most part, unsatisfactory as a kidney substitute.

Most investigators have employed an isotonic perfusion fluid. The effects of forced intestinal dialysis and the beneficial possibilities which might be derived from the use of hypertonic enteric perfusion fluids have been recognized but not fully exploited. Goudsmit[14] employed a hypertonic solution of sodium sulfate. He found that the average concentration of urea in the drained fluid was 93 per cent that of the blood in the normal subjects and 75 per cent in the uremic patients. He stated that these results compared favorably with those observed in peritoneal lavage fluids and suggested that continuous lavage over a longer period of time would be useful as a renal substitute. White and Harkins achieved best results by utilizing a hypertonic 3 per cent saline solution alone or with 3 per cent sodium sulfate.[29] Kolff[15] found that by the addition of $MgSO_4$ to the perfusion fluid, composed of NaCl, $NaHCO_3$, KCl, and dextrose, his results were improved, presumably by diminishing the rate of absorption of the fluid. These observations, together with the belief that the effects of forced intestinal drainage with various molecular concentrations had not been fully investigated, prompted the following experimental work.

The purpose of this investigation was twofold: Part I, to select a suitable hypertonic enteric perfusion fluid; Part II, to evaluate the therapeutic effect of continuous hypertonic isolated loop lavage in the nephrectomized dog.

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PART I

MATERIAL AND METHODS

A. Preparation of the Animals.—Six healthy mongrel dogs weighing from 5.9 to 15.4 kilograms were selected. Under Nembutal anesthesia an arbitrarily chosen 100 cm. segment of small bowel was isolated. Measurements were taken from a point 10 to 15 cm. from the ileocecal valve in an oral direction. Thus, the loop was essentially ileum and a portion of the jejunum. The ends of the isolated segment were exteriorized as a double stoma loop enterostomy (Thiery-Vella fistula[26]). The continuity of the remaining gastrointestinal tract was re-established by a side-to-side anastomosis or by an oblique end-to-end method.[8] Following a convalescent period of three to six weeks, Foley bag catheters were introduced into the intestinal stomata and inflated. The animals were weighed before and after each period of perfusion. During the intestinal irrigation period the animals were placed, unrestrained, on a table, or were lightly tethered in the dorsal position on a dog board. Nembutal sedation was generally not required. Throughout the duration of this work the dogs were maintained on the usual kennel diet. During the experiments no food or liquid was offered.

B. Perfusion Fluid.—Tyrode solution containing the following anhydrous salts per liter was employed as a basic perfusion fluid: NaCl, 8.0 Gm.; HCl, 0.2 Gm.; $CaCl_2$, 0.1 Gm.; $MgCl_2$, 0.1 Gm.; NaH_2PO_4, 0.06 Gm.; $NaHCO_3$, 1.0 Gm.; and dextrose, 1.5 grams. The irrigating fluid was not sterile. Hypertonic solutions were made by adding sufficient $MgSO_4$ to Tyrode's basic formula to make additional 1 per cent, 2 per cent, 10 per cent, and 20 per cent concentrations. In other experiments, hypertonic Tyrode solution was made by adding enough sucrose to make a 20 per cent sugar solution.

C. Method of Perfusion.—An 18 liter carboy containing the irrigating solution was placed two and one-half to three feet above the level of the dog. From this, the fluid flowed by siphonage through rubber and glass tubes to the inflated Foley catheter. Interposed within this system was a graduated burette and a drip bulb to allow adjustment and observation of the rate of flow. A flow of 20 to 50 ml. per minute was arbitrarily chosen, because this rate was found most effective for urea clearance by peritoneal lavage in experimental animals.[22] A coiled glass tube through which flowed irrigating fluid was placed in a water bath thermostatically controlled to maintain the fluid at body temperature. The inflated Foley catheter within the distal intestinal stoma was connected by rubber tubing to a 4 liter bottle containing some toluol. Flow through the dog's loop was maintained by siphon. The perfusion period varied from three to four hours.

D. Chemical Determinations.—Heparinized samples of blood were drawn both before and after perfusion. The nonprotein nitrogen was determined by the method of Rappaport and Eichhorn.[21] Plasma chloride was calculated[23] in all experiments. Plasma total base values were determined[25] as a concentration index of sodium, potassium, magnesium, and calcium. Total plasma proteins were measured by the method of Lowry and Hunter.[17] Blood hematocrits were determined after a modified Wintrobe technique.[16]

Catheterized specimens of urine were obtained both before and after perfusion and the following determinations carried out: specific gravity, nonprotein nitrogen, total protein, chlorides, and total base.[11] The methods of determination were adaptations of those employed in the case of heparinized blood samples.

The recovered perfusion fluid was volumetrically measured and similar chemical determinations were made. In certain experiments (Part II), urea values of the recovered perfusion fluid were calculated after the gravimetric method of Allen and Luck.[2]

RESULTS

Under standard conditions, twenty-eight intestinal perfusion experiments were carried out. The term "standard conditions" refers to perfusion of a given fluid at a rate of flow of 20 ml. to 50 ml. per minute through 100 cm. loops of isolated small bowel for a period of three to four hours.

Tyrode solution containing 20 per cent sucrose by volume extracted an average 52.1 mg. per cent nonprotein nitrogen in three to four hours by isolated loop perfusion, or more than six times the quantity recovered in any other hypertonic perfusion experiment and ten times the amount recovered with an isotonic solution (Fig. 1). The quantity of nonprotein nitrogen extracted in the perfusate was roughly directly proportional to the hypertonicity of the

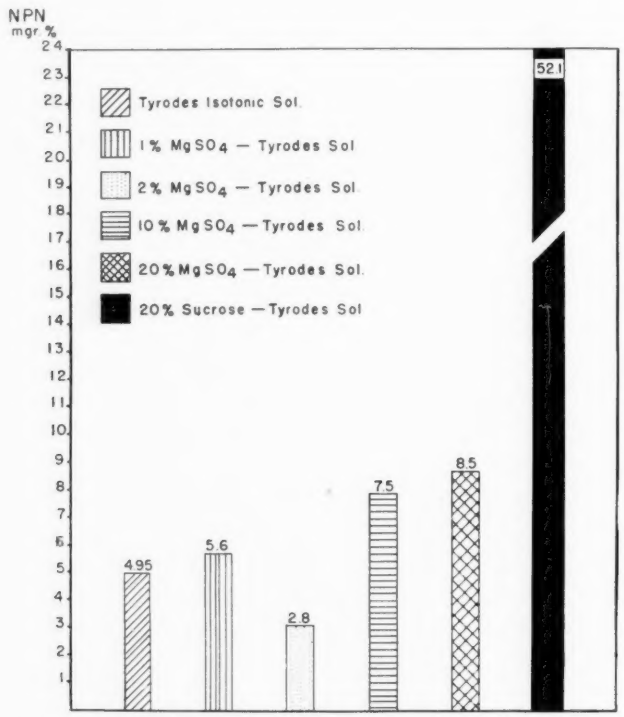

Fig. 1.—Nonprotein nitrogen recovered in perfusate using Tyrodehypertonic solutions under standard conditions.

perfusion solution (Fig. 1). Enteric perfusion using Tyrode's isotonic solution produced the greatest reduction of plasma nonprotein nitrogen (Fig. 2). No correlation between the degree of plasma nonprotein nitrogen reduction and the amount of nonprotein nitrogen extracted in the perfusate could be established (Figs. 1 and 2). Individual variations of protein metabolism, state of hydration, and nonprotein nitrogen-cellular-interstitial space-vascular equilibrium may account for these discrepancies.

Tyrode solution containing 20 per cent $MgSO_4$ reduced plasma nonprotein nitrogen rapidly. Dehydration, salivation, vomiting, apparent disorientation, and exhaustion prompted perfusion discontinuance after one and one-half hour respectively. Eight hours later, both dogs had fully recovered.

Isotonic and hypertonic Tyrode sucrose solutions containing 0.001M $HgCl_2$ as a protoplasmic poison extracted nonprotein nitrogen in comparable quantity to Tyrode sucrose solutions without the protoplasmic poison (Fig. 3).

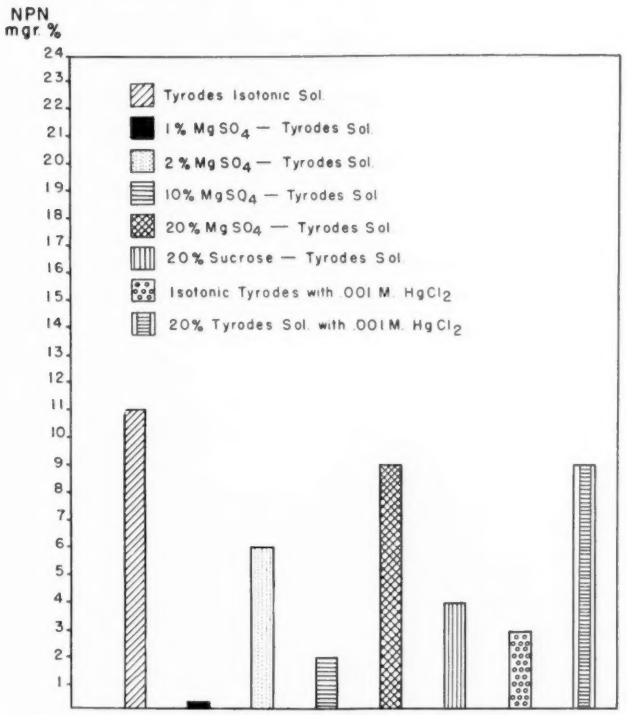
NPN
mgr. %
Tyrodes Isotonic Sol.
1% MgSO4 — Tyrodes Sol.
2% MgSO4 — Tyrodes Sol.
10% MgSO4 — Tyrodes Sol.
20% MgSO4 — Tyrodes Sol.
20% Sucrose — Tyrodes Sol.
Isotonic Tyrodes with .001 M. HgCl2
20% Tyrodes Sol. with .001 M. HgCl2

Fig. 2.—Plasma nonprotein nitrogen loss following isolated loop perfusion with hypertonic Tyrode solution under standard conditions.

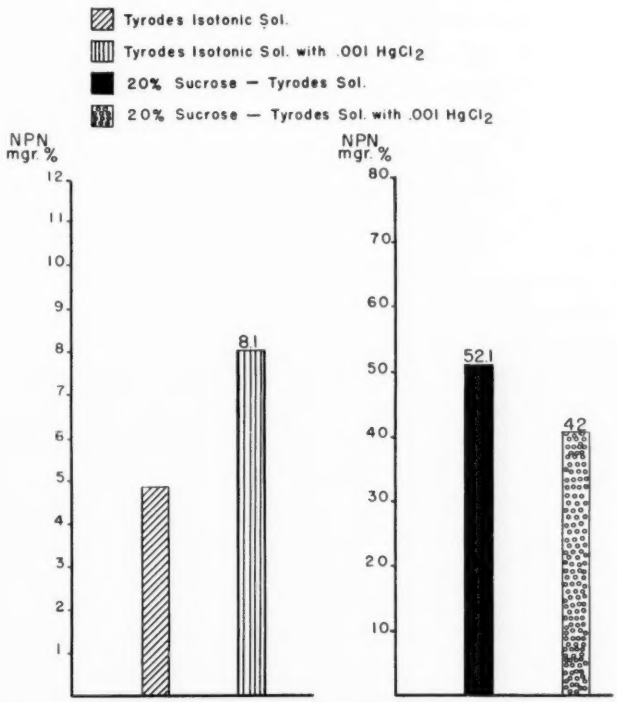

Fig. 3.—The effect of perfusion fluids containing a protoplasmic poison on the quantity of nonprotein nitrogen recovered (standard conditions).

No constant changes were observed in weight, hematocrit, chloride, or total base values following isolated loop perfusion with hypertonic sucrose-Tyrode solutions. No general edema nor evidence of hyperchloremic acidosis was encountered during the reported experiments.

Perfusate collected from dogs irrigated with Tyrode hypertonic sucrose fluids gave a negative benzidine occult blood reaction. Microscopic examination showed no significant cellular structures after three to four hours of perfusion.

Increased urine concentration was observed in the majority of experiments, although corresponding weight changes and hematocrit values failed to reflect dehydration. Urine qualitative reduction tests yielded a ++ to +++ reaction in animals perfused with a Tyrode 20 per cent sucrose solution.

DISCUSSION

Twenty per cent hypertonic sucrose-Tyrode solution was the most effective perfusion fluid in removing nonprotein nitrogen from the experimental animal. Wells and Johnson[28] have suggested that hypertonic solutions increase the excretion through the bowel wall by producing a chemical or physical irritation rather than by a purely osmotic process. In order that the electrolytic balance of the experimental animal be preserved, and since the process of dialysis is reversible, an irrigating fluid (Tyrode solution) closely resembling the electrolyte pattern of the blood was chosen. Distilled water alone as a perfusion fluid destroys bowel mucosa.[9] Electrolyte balance, particularly with reference to the concentration of the monovalent ions, is not entirely dependent on the action of dialysis alone. Visscher[30] has shown that monovalent chloride ions may pass from the bowel lumen by selective action of the mucosa into the circulating blood stream against higher concentration gradients. Comparable clinical observations have been noted by Daugherty and co-workers.[6] Apparent alterations in the electrolyte patterns may be caused by changes in hydration of the experimental animal as well. There was no significant constant change in hemoconcentration as measured by hematocrit values, fluid balance, or electrolyte equilibrium during the perfusion period.

$MgSO_4$ in concentrations greater than 10 per cent is not satisfactorily tolerated for prolonged periods of perfusion. Greater molecular concentrations of relatively nontoxic molecules in an electrolytically isotonic solution appeared to us to offer a solution for a more effective perfusion fluid. Sucrose was chosen because of its (1) low molecular weight, (2) low toxicity to living tissue, (3) potential caloric value if hydrolyzed and absorbed, (4) availability, and (5) low cost.

With concentrations of sucrose of 50 per cent, vomiting and other toxic manifestations occur. Sucrose in hypertonic solution is damaging to cell structure.[7] It is reasonable to expect that with prolonged irrigation the effect of intestinal perfusion, as measured by the recovery of nonprotein nitrogen might decrease. However, by damaging the action of the mucous membrane with a mercuric chloride 0.001 molar solution[30] in Tyrode solution for perfusion purposes, comparable amounts of nonprotein nitrogen were recovered as compared

with those experiments where isotonic Tyrode solution or 20 per cent sucrose-Tyrode solution was used alone. This suggests that the state of physiologic integrity of a bowel wall will not greatly affect dialysis.

Dogs perfused with 20 per cent $MgSO_4$-Tyrode solution and other high concentrations of hypertonic fluids were able to extract water from the perfusion fluid. In both experiments in which 20 per cent $MgSO_4$-Tyrode solution was employed as a perfusion fluid, the recovered volume of irrigating solution was less than the volume introduced. Comparable observations have been made by White and Harkins.[29]

Part II

MATERIALS AND METHODS

The animals utilized in Part I were bilaterally nephrectomized under sodium pentothal anesthesia. Twenty-four hours after operation, continuous irrigation of the Thiery-Vella loop was instituted with 20 per cent sucrose-Tyrode solution modified after the methods employed in Part I. Water, but no food, was offered during the continuous perfusion period. Intramuscular divided doses of sodium pentothal were administered as a sedative. Parenteral fluids and salts were given intravenously as required, based on plasma studies as outlined in Part I.

The following determinations were carried out before nephrectomy and every twenty-four hours thereafter until the animal expired: hematocrit, plasma chlorides, total protein, nonprotein nitrogen, and total base. The recovered perfusate was volumetrically measured and examined for urea, nonprotein nitrogen, chlorides, total protein, total base, occult blood, and fragments of tissue. The period of survival was measured from the conclusion of the nephrectomy operation until the animals expired.

RESULTS

Average survival time of nephrectomized dogs treated by continuous hypertonic (20 per cent sucrose-Tyrode solution) isolated loop irrigation was 80.1 hours. In spite of 4.38 to 6.12 Gm. urea recovered in the twenty-four hour periods, the plasma nonprotein nitrogen progressively increased (Fig. 4). The typical clinical picture of uremia characterized by anorexia, vomiting, excitatory phase, spasmodic muscular contractions, apparent irrationality, lethargy, and death was observed. There was no evidence of edema. All animals lost from 3 to 5 pounds of weight accounted for by (1) vomiting, (2) salivation, (3) insensible water loss, (4) fecal incontinence. Two hundred to five hundred milliliters intravenous fluids per animal per twenty-four hours were sufficient to maintain normal hematocrit values. Total plasma proteins and chloride values remained relatively constant except as temporarily affected by the animal's state of hydration.

Perfusion fluid recovered (24.4 liters per twenty-four hours, average volume) yielded 0.2 Gm. urea per liter or 4.87 Gm. per twenty-four hours (Fig. 4). Occult blood was detected after forty-eight hours of continuous lavage, and fragments of tissue were seen in the perfusate after seventy-two hours.

Post-mortem studies revealed no pleural but slight (15 to 20 ml.) peritoneal effusion. The Thiery-Vella loops (average length, 82 cm.) contained multiple mucosal ulcerations from 1 mm. to 4 cm. in diameter. In two speci-

mens, the isolated loop walls were thickened two to three times normal size by edema and hyperemia. Subserosal hyperemia was pronounced. The amount of bowel damage was directly proportional to the duration of perfusion.

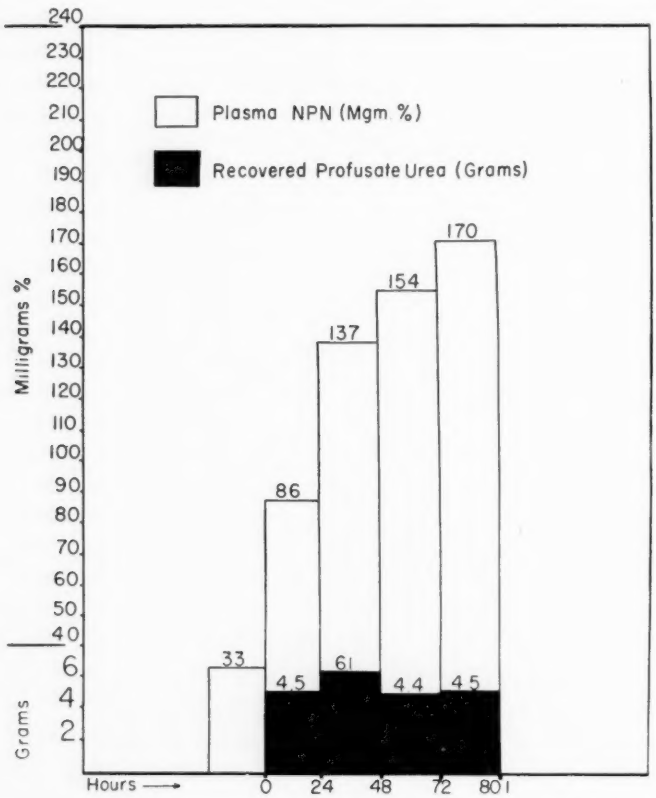

Fig. 4.—Relationship of plasma nonprotein nitrogen to recovered urea by continuous hypertonic lavage (20 per cent sucrose-Tyrode solution) in nephrectomized dogs. (Average values, five experiments.)

DISCUSSION

Survival time (eighty hours) of nephrectomized dogs treated by continuous hypertonic (20 per cent sucrose-Tyrode solution) perfusion was insignificantly longer than that of the controls of Bliss and co-workers[4] (three days) and White and Harkins[29] (seventy-three hours) and was comparable to that of the controls of Abbott and Shea[1] (three to five days). Survival time of untreated nephrectomized dogs is variable. Ivy, Barry, and co-workers had control animals survive for more than one hundred hours average.[3, 10, 18]

On the basis of comparative survival times of the untreated nephrectomized dogs, any claim of therapeutic success by the method studied must be regarded as invalid.

Hypertonic isolated loop enteric lavage yielded significant quantities of urea in spite of extensive mucosal damage, a fact foreshadowed (Part I) by the

relatively large nonprotein nitrogen yield through the isolated bowel wall, the mucosa of which was damaged by a protoplasmic poison (Fig. 3).

It was not within the scope of this work to observe, in great detail, the effect of hypertonic isolated loop lavage upon the chemical structure of the plasma or extracellular fluid. However, from our data, little deleterious effect was observed upon the normal values of proteins, chlorides, total base, and hematocrit. Prolonged continuous irrigation produced mild dehydration readily corrected by intravenous fluid replacement.

We are in accord with Gamble's belief[13] that "in uremia, the defense of the chemical structure of extracellular fluid is of much more importance from the point of view of survival than the reduction of azotemia." Recent work[24] has strengthened this belief.

Therefore, it would seem reasonable that a more promising approach to the therapy of uremia would be a combination of azotemic reduction and proper administration of a balanced solution of electrolytes.

SUMMARY AND CONCLUSIONS, PARTS I AND II

1. Hypertonic (20 per cent sucrose-Tyrode) perfusion fluid was ten times more effective than isotonic fluid (Tyrode) in extracting nonprotein nitrogen from a dog's isolated enteric loop.

2. The amount of nonprotein nitrogen extracted by isolated enteric lavage was roughly proportional to the hypertonicity of the perfusion fluid.

3. Hypertonic (20 per cent sucrose-Tyrode) solution for isolated enteric loop lavage produced no appreciable changes of plasma acid-base or water equilibrium over three- to four-hour periods.

4. Continuous hypertonic (20 per cent sucrose-Tyrode) isolated enteric lavage did not prolong the survival period of nephrectomized dogs.

5. Continuous hypertonic (20 per cent sucrose-Tyrode) enteric lavage eventually produces mucosal ulcerations and bowel damage.

The authors are indebted to Dr. D. A. MacFadyen for his many helpful suggestions and to Miss Helen Ellis for her technical assistance in this study.

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PROGRESSIVE CHANGES IN LIVER COMPOSITION, FUNCTION, BODY FLUIDS, AND LIVER CYTOLOGY DURING PROTEIN DEPLETION IN THE RAT AND THE EFFECT OF CHOLINE UPON THESE CHANGES

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IT IS known that the liver is the first organ to suffer a serious loss of protein during starvation or the development of protein deficiency. Addis, Poo, and Lew[1, 2] found, for example, that 20 per cent of the protein of the liver of well-fed rats was lost during a two-day fast and that 40 per cent was lost after a week of fasting. Kosterlitz[3, 4] reports that rats fed a protein-free diet lost 15 per cent of the liver cytoplasm during the first day. It thus appears that a considerable amount of the liver protein is easily mobilized. After the first few days the liver losses become much less and, according to Campbell and Kosterlitz,[5] follow an exponential curve as protein starvation continues.

Cytologic evidence has shown that the ribonucleic acids also are rapidly lost from the liver during protein deficiency. Elman and associates,[6, 7] Kosterlitz,[3, 4] and Wang and Hegsted[8] observed simultaneous clearing and vacuolization of the cytoplasm together with losses of the basophilic staining material which was apparently ribonucleic acid.[9, 10, 11] It is not clear whether or not these losses represent serious functional changes of the liver.

An accumulation of fat in the liver has been observed during the development of protein deficiency.[4, 6, 12] In view of the diets which have been used, it may be expected that this may be partially related at least to a simultaneous deficiency of choline. Hough and co-workers[13] observed that the administration of choline to protein-depleted dogs largely prevented the changes in hepatic dye clearance and the rise in serum phosphatase which occurred on the protein-deficient diet they were using. It may be expected that the development of protein deficiency in man may also be complicated by low choline intakes.

This paper reports the results of studies of protein deficiency in rats which received diets either poor or rich in choline. Changes in the composition of the liver, blood and plasma volumes, thiocyanate space, bromsulfalein clearance, and plasma nitrogen were determined and histologic examinations of the livers were made in an attempt to correlate morphology with the analytic data obtained.

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EXPERIMENTAL

The study was made with young adult female rats which weighed approximately 200 grams at the time they were given the protein-free diet. The diets were of the purified type previously described[8] and contained glucose, 89 per cent, corn oil, 5 per cent, salt mixture,[14] 4 per cent, cod-liver oil, 2 per cent, and supposedly adequate levels of all of the B complex vitamins except choline in the low choline diet. This diet contained 30 mg. of choline chloride per 100 Gm., while the high choline diet contained 500 mg. per 100 grams. The animals were housed in individual cages in a room of approximately constant temperature, 78° to 80° F. The diets were fed ad libitum but food intake was recorded at two- or three-day intervals by weighing the amount of food not eaten. Animals were killed at intervals of three, twelve, twenty-five, or more days as indicated below. A total of thirty animals was started upon each diet but it was not possible to make all measurements on each animal. The figures presented below show the number of animals from which data were obtained at each period of deficiency.

At the time of sacrifice, the blood and plasma volumes, thiocyanate space, and bromsulfalein clearance were determined as previously described.[15] The liver was immediately removed; samples were taken from both the right and left lobes for histologic study; and the remainder was prepared for chemical analysis. Water content was determined by drying at 95° C., glycogen and fatty acids plus the unsaponifiable fraction as previously described,[8] and total nitrogen by the macro-Kjeldahl method. Total plasma nitrogen was measured colorimetrically by the Nessler method after digestion.

RESULTS

Biochemical Analysis.—Fig. 1 summarizes the changes observed in the liver weight. On the high choline-low protein diet there was an immediate fall during the first three days from approximately 8 grams to about 5.5 grams. This weight was maintained approximately constant for about two weeks and then gradually fell. When expressed as per cent of body weight the original decrement is still apparent, but after a period of depletion it again regained the original relation to body weight and was maintained at this level throughout the depletion. These changes are interpreted as an original loss of liver substance which is much greater than the relative loss of body substances, but during the following two weeks the relative loss of body weight exceeded that of the liver. During the latter stages of depletion the percentage losses from liver and the remainder of the body are approximately equal.

Animals which received the low choline diet failed to show the original liver weight loss because of the accumulation of fat as shown below. During later periods most, but not all, of the fat was lost so that the liver weight changes were approximately the same as in the high choline group. This was also shown by the similarity of the two groups when fat and glycogen were subtracted from the dry liver weight to give nonfat, nonglycogen liver weight, presumably protein weight. This fell gradually and approximately to the same extent in both groups.

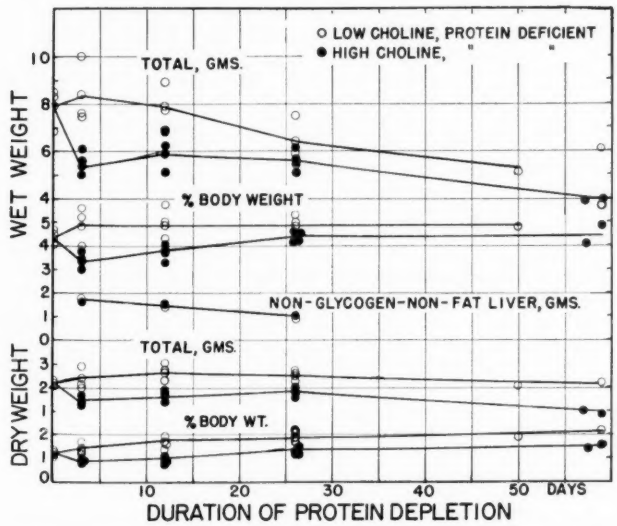

Fig. 1.—Changes in liver weight with respect to time on protein-free diets.

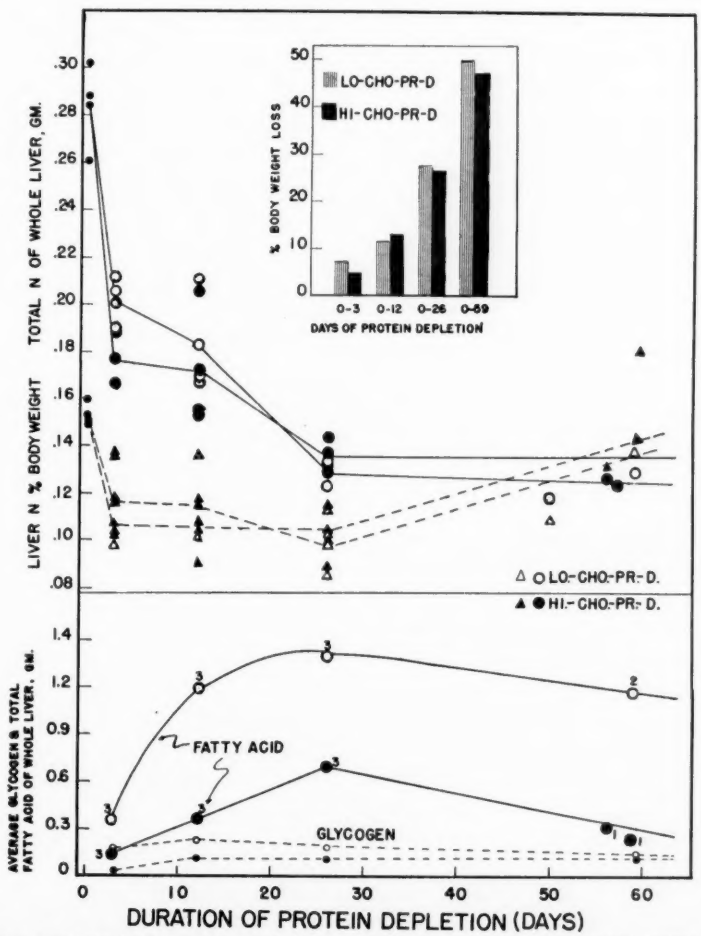

Fig. 2.—Changes in liver nitrogen, fat, and glycogen, and body weight losses with respect to time in rats receiving high and low choline, protein-free diets. Numbers in the lower part of the figure indicate the number of livers analyzed.

The loss of nitrogen from the liver was similar regardless of the choline content of the diet (Fig. 2). Body weight loss was also similar in the two groups (Fig. 2, insert). In the latter stages of deficiency the rate of body weight loss exceeded that of liver nitrogen loss. Some fat accumulated in the liver during the early stages of deficiency even though choline was supplied. Consistently larger amounts of glycogen were found in the livers of animals which received the lower level of choline.

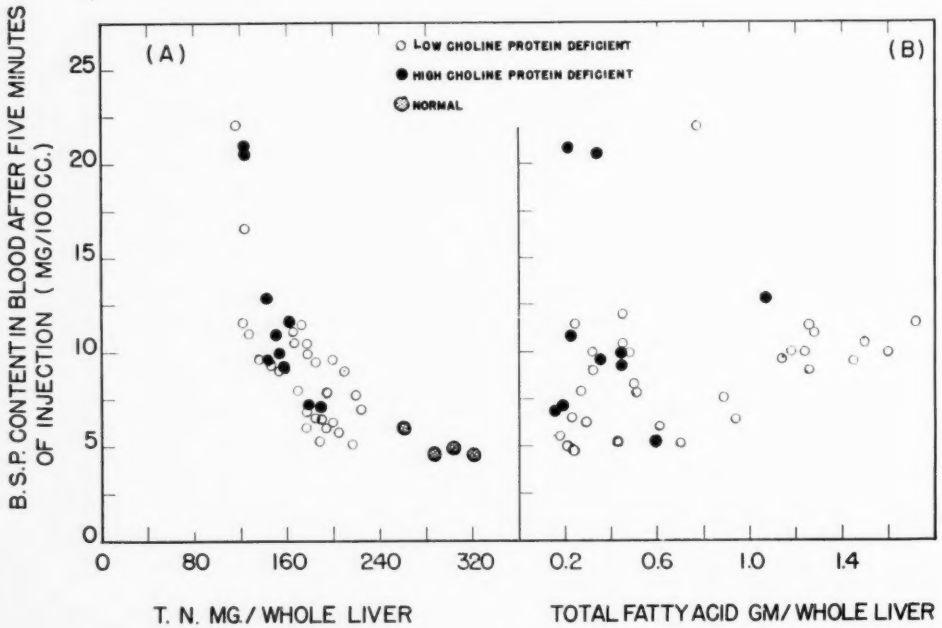

Fig. 3.—Scatter diagram showing the relation of blood bromsulfalein concentration five minutes after injection to total liver nitrogen as compared with total liver fat. The apparent correlation with liver nitrogen is mainly due to a simple decrease in liver size with loss of weight.

The absolute amount of bromsulfalein cleared from the blood fell markedly during the depletion period. This may be inferred from Fig. 3 since the blood concentration of bromsulfalein five minutes after the injection of a 5 mg. dose is much higher in those animals with less total nitrogen per liver, that is, the animals subjected to protein deficiency for the longest period of time. It is also clear from the right-hand side of the same figure that the blood level is not well correlated with the degree of fatty infiltration which developed in these animals. Thus as protein deficiency developed, the animals were able to remove smaller amounts of a standard dose of bromsulfalein from the blood in a given time period. However, the deficiency is not only associated with a decrease in liver size but with a fall in body weight and blood volume as well, and an adequate representation of the situation must take these changes into account. Results presented earlier[15] showed that the bromsulfalein clearance of normal rats in the first five minutes after injections yielded a straight-line relationship with dosage

when both clearance and dosage were expressed in terms of milligrams per 100 grams of body weight. The values obtained with the protein-depleted animals were therefore compared with the values expected from normal rats of the same weight. The deviations from normal (negative deviation equals increased bromsulfalein retention) are shown in Table I. In none of the animals was the deviation from the expected value great, and none of the mean values presented in Table I were significantly different from the expected value when tested statistically by the "t" test. Thus it is concluded that essentially all the decrease in bromsulfalein clearance is accounted for by the loss of body and liver weight, and there is no proof of malfunction of the remaining liver tissue. It is true that the deviations from normal tend to become greater as the deficiency is prolonged, and additional data might prove these deviations significant statistically.

TABLE I. BROMSULFALEIN CLEARANCE IN PROTEIN-DEFICIENT ANIMALS COMPARED WITH VALUES FOR NORMAL RATS

DIET	TIME ON DEFICIENT DIET	NUMBER OF ANIMALS	MEAN DEVIATION OF BROMSULFALEIN CLEARANCE FROM NORMAL (MG./100 GM. BODY WEIGHT)
High choline, protein-free	3	9	−.061 ± .024*
	12	10	−.149 ± .096
	25	7	−.187 ± .095
	40	2	−.225 ± .0087
	56	2	−.370 ± .322
Low choline, protein-free	3	7	−.076 ± .157
	12	9	−.181 ± .137
	24	7	−.092 ± .296
	50	1	−.40

*Standard error of the mean.

The changes in the body fluid volumes are indicated in Fig. 4. The thiocyanate space fell gradually, and nearly parallel to the fall in body weight, so that the ratio of thiocyanate space to total body weight remained constant for approximately forty days. After this period there appeared to be a slight tendency for the thiocyanate space per unit body weight to increase. This increase, if significant, would represent a tendency toward edema. The changes in plasma volume showed the same trend as those of the thiocyanate space so that the ratio, $\frac{\text{ECFV}}{\text{PV}}$, remained essentially constant even after prolonged depletion.

The total plasma nitrogen concentration showed an early marked drop at the same time that the plasma volume was shrinking. This resulted in a considerable decrease in the total circulating plasma nitrogen (TCPN). The loss in body weight was also large (Fig. 2, insert) and the total circulating plasma nitrogen per 100 grams of body weight showed only a slight and apparently insignificant drop. During the latter stages of the deficiency, the plasma nitrogen concentration increased considerably so that the total circulating nitrogen per 100 grams of body weight rose. This occurred simultaneously with the rise in thiocyanate space. It is clear that there was no consistent relation between the plasma nitrogen and the thiocyanate space. Since the formation of edema

is expected to be related to a fall in plasma protein concentration, the changes observed are the opposite of those expected. The total plasma nitrogen figures reflect changes in both plasma proteins and nonprotein nitrogen. Since the

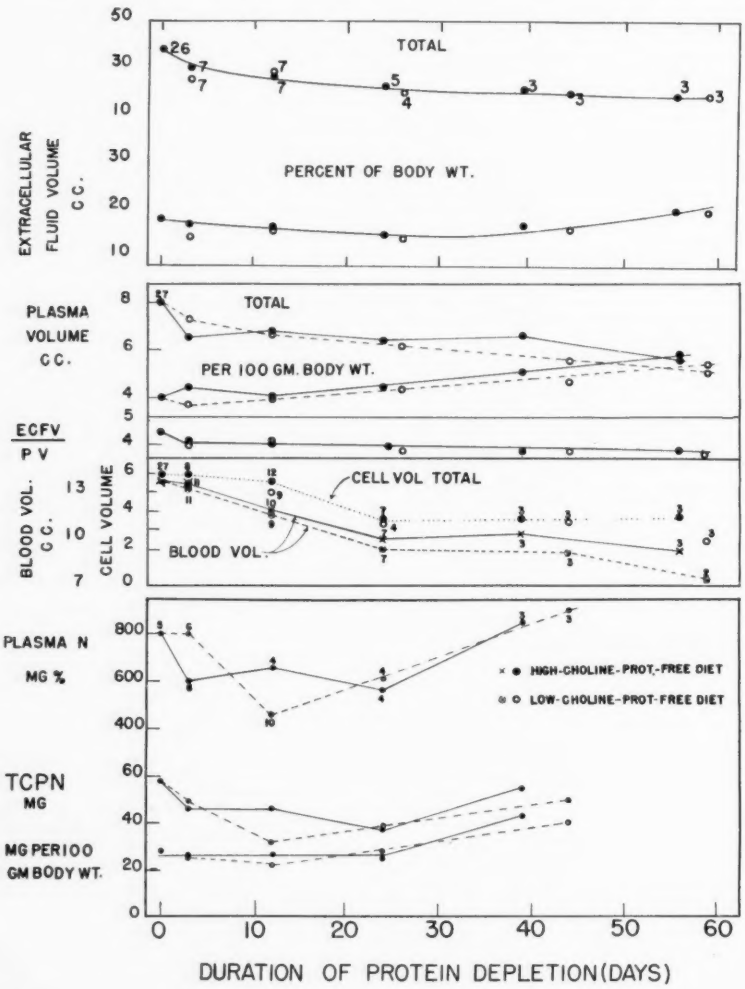

Fig. 4.—Variations in the distribution of body fluid volumes and plasma nitrogen during protein depletion. Numbers at the various points indicate the number of animals used.

nonprotein nitrogen of typical animals at various stages of depletion remained essentially constant, and less than 30 mg. per cent, it is apparent that an increase in plasma nitrogen represents an increase in the total plasma proteins.

None of the data in Fig. 4 indicates that choline deficiency and the resultant fatty livers are of importance in the changes in fluid volumes observed.

Gross Pathology.—As early as the third day, some of the livers appeared paler than those of normal animals. As the deficiency period increased, the livers became successively paler and yellower until at twenty days of deficiency they were definitely and uniformly yellowish-brown rather than the normal

purplish-red color. After twelve days on the deficient diet, some animals showed marked involution of the thymus. From this time on, subcutaneous edema appeared in a few of the animals. Although difficult to evaluate because of the great loss of tissue and fat, this appeared more severe and more uniform after long periods of deficiency.

Microscopic Pathology.—Blocks of tissue were fixed in 4 per cent aqueous formaldehyde, Zenker-acetic solution, and absolute alcohol. Eosin-methylene blue, hematoxylin and eosin, Best carmine, and sudan IV stains were made.

The histologic changes observed were similar to those described by Elman, Smith, and Sachar,[7] Wachstein,[16] Kosterlitz,[4] and Kosterlitz and Campbell,[5] and, with the exceptions noted below, they were essentially the same in both the high and low choline diets. (Fig. 5.) The earliest change consisted of enlargement of the liver cells at the expense of the sinusoids, especially in the peripheral zones of the lobules. The sinusoids were compressed and largely obliterated after a few days on the deficient diet. Simultaneously the cytoplasm became less dense ("cytoplasmic clearing") and was represented by a few coarse eosinophilic or basophilic strands. There was an apparent decrease in basophilic granules in the cytoplasm which have been demonstrated to be composed of ribonucleic acid by Caspersson and Schultz and others.[9-11] The remaining basophilic granules appeared to have been displaced toward the cell membrane which stained deeply and conspicuously ("cytoplasmic rimming").

Whereas in normal animals the nuclei of liver cells were round, vesicular, and uniform in size with finely granular and evenly distributed chromatin, the nuclei of some of the liver cells of rats on the deficient diets were variable in size and shape and tended to be hyperchromatic. These changes were not necessarily more pronounced in those cells showing the cytoplasmic changes described. Also as early as the third day, fat in the form of large or small droplets appeared in the liver cells of the periportal areas. This was more marked in the animals receiving the low choline diet. Best carmine stains also indicated an increase in glycogen, mainly in the periportal cells, but the zonal distribution was not as marked as that of fat.

These changes, typical of all the animals on the low protein diets, were more marked in the low choline than in the high choline group. In Table II the various changes have been graded and are listed at different stages of the experiment. In general the most striking changes were consistently observed in animals depleted for twelve to twenty-six days. In a few of the low choline animals, however, cytoplasmic clearing and rimming of equal intensity was found as early as three days. Typically these livers appeared as a mosaic of polygonal, clear cells with central nuclei. In the few animals that were sacrificed as late as fifty days, it was noted that in the low choline, protein-free group the findings of markedly increased cell size, cytoplasmic clearing and rimming were the same as in the twelve and twenty-six day groups. A moderate amount of fat was found centrally in the lobules, differing from the periportal distribution noted earlier. There was also considerable variation in the size and staining quality of the nuclei. Many cells were multinucleated. In the animals receiving the high choline, low protein diet, the changes were similar except that they

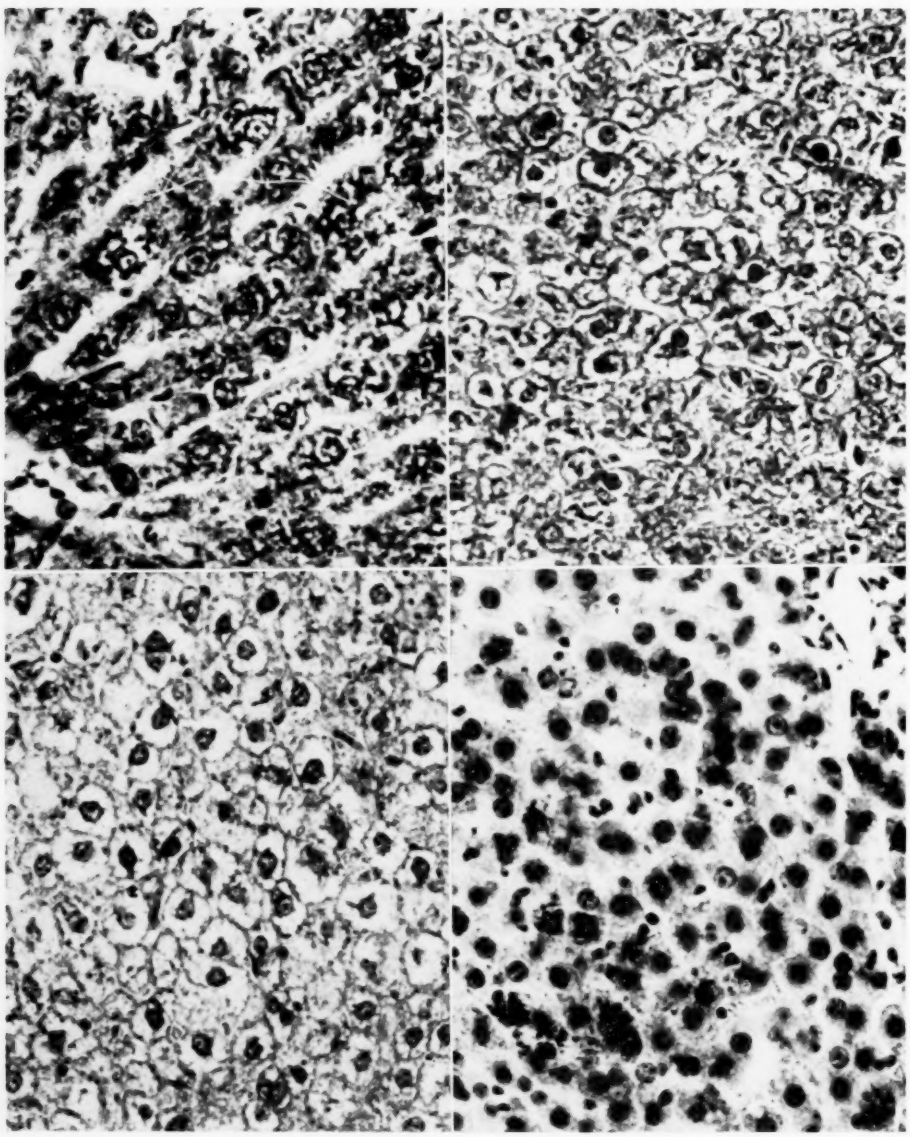

Fig. 5.—Liver changes with protein depletion. *A*, Normal rat (eosin-methylene blue, ×350); *B*, three days of depletion (eosin-methylene blue, ×350); *C*, twenty-six days of depletion (eosin-methylene blue, ×350); *D*, seventy-six days of depletion (sudan IV frozen section, ×350). As early as three days there is marked rarefaction of the cytoplasm with the remaining basophilic material chiefly at the edges of the cells. The sinusoids have been largely obliterated. At twenty-six days the liver appears as a mosaic of rarefied polygonal cells, but by seventy-six days (high choline, protein-free animal) the appearance approaches that found in the well-nourished animal with slight to moderate fatty infiltration chiefly central in location.

became less marked as the depletion was prolonged. A few animals survived as long as seventy-six days and in these the cell size was essentially normal and there was no cytoplasmic clearing or rimming. The fat which persisted tended to be centrally or variably dispersed in the lobules. Nuclei were variable in size and shape.

TABLE II. SUMMARY OF THE HISTOPATHOLOGIC CHANGES IN THE LIVER AFTER VARIOUS PERIODS ON THE PROTEIN-DEFICIENT DIETS

TIME ON DEFICIENT DIET (DAYS)	CYTOPLASMIC CLEARING		CYTOPLASMIC RIMMING		FAT QUALITY		FAT DISTRIBUTION		NUCLEUS		CELL SIZE	
	HIGH CHOLINE	LOW CHOLINE	HIGH CHOLINE	LOW CHOLINE	HIGH CHOLINE	LOW CHOLINE	HIGH CHOLINE	LOW CHOLINE	HIGH CHOLINE	LOW CHOLINE	HIGH CHOLINE	LOW CHOLINE
3	0	++ to +++	0	++ to +++	0 to +	+	Periportal	Periportal	Normal	Normal	0	+
12	+ to ++ (Variable)	++++	0 to -	+++	0 to ++	+++	Periportal, but some loaded cells throughout	Universal	Normal or slightly hyper-chromatic	Normal	±	+
25	+ to ++	++ to +++ (Variable)	0 to -	++ to +++	+ to +++	++ to ++++	Periportal	Periportal	Variable in size and stain	Hyper-chromatic	±	+
50		+++ (Variable)		++ (Variable)		++		Central		Variable in size and stain		+
76	0		0		+ to ++		Central and variable		Variable in size and stain		0	

0, No change from normal; the number of plus signs indicates the relative extent of the changes observed.

DISCUSSION

In general the results of these studies agree with previous investigations.[4, 6] The cytologic evidence and the chemical findings both show marked changes in the liver during early protein deficiency. Early in the period of deficiency the large losses of protein are accompanied by a considerable accumulation of fat. The fatty infiltration is apparently not completely preventable by choline in the amounts used, although larger amounts might prove more effective. Glycogen accumulates at the same time and to a greater degree in the livers of the choline-deficient animals.

During later periods, when the deficiency is greater, the nitrogen losses from the liver are less and a large proportion of the loss is from the other body tissues. The ratio liver weight/total body weight actually increases at this time. It may be significant that the food intake of the animals is fairly well maintained for three or four weeks upon the diet, but then begins to diminish. During the latter stages of the deficiency there may be a caloric as well as a protein deficiency. The present data are insufficient to evaluate this aspect of the problem, however, since nothing is known of the metabolic rates or caloric requirements of severely deficient animals. It may be expected that the metabolic rate has declined if the animals behave as in starvation, and this together with a marked loss in body weight should lower the caloric needs. However this may be, the evidence is clear that after severe depletion there is a return toward normality in liver structure both chemically and histologically, and this is aided and increased by the presence of choline. There is apparently no basis for deciding whether this is advantageous for the animal, at least from this study. Choline obviously cannot replace protein in the diet, and the animals, regardless of the presence or absence of choline, lose weight, become emaciated, and eventually die. There also does not appear to be any great change in over-all nitrogen metabolism as the result of the degree of fatty infiltration of the liver which occurred on the diets used.

The absolute clearance of bromsulfalein per rat falls markedly during the depletion period, but the clearance per unit body weight is not significantly less than normal. It therefore appears that the total ability of the liver to remove bromsulfalein is greatly changed by the decrease in size, but the function per gram of liver tissue remains nearly normal. This finding is somewhat at variance with the results of previous studies with dogs. In the experience of Hough and Freeman and co-workers,[12, 13] dogs fed a low protein diet soon showed high serum phosphatase values and decreased rates of clearance of rose bengal. However, their basal diet probably contained less choline than our low choline diet. Most of the changes observed by these authors were prevented by the feeding of choline and therefore were due primarily to choline deficiency. McKibbin and associates[17] found lowered bromsulfalein clearance in choline-deficient puppies. Similar results also were observed in choline-deficient rats by Maclean, Ridout, and Best,[18] but only in those animals with very large amounts of liver fat. One may conclude that changes in dye clearance per unit body or liver weight due to protein deficiency are relatively slight and are detectable only after relatively severe depletion. On the other hand, unless

choline is specifically added, most low protein diets will also be low in choline and this may be the primary factor in lowering the rate of dye removal. The accumulation of fat, however, must be relatively great before it is effective in changing the bromsulfalein clearance in rats.

The modifications in the volumes of the body fluids will receive more attention in a following paper in which the effect of low temperatures has been studied in conjunction with protein deficiency. It may be noted, however, that (a) the tendency toward edema as indicated by an increase in thiocyanate space relative to body weight occurred only after very severe protein depletion and was then only slight, (b) the plasma volume decreased approximately equally with body weight and the ratio of thiocyanate space to plasma volume remained nearly constant during the depletion, and (c) although there was a marked decrease in the plasma nitrogen on the deficient diet, this not only showed no relation to the tendency toward edema but actually was higher at the time when the thiocyanate space tended to increase. This lack of correlation between plasma proteins and edema has been described by Keys and associates in human volunteers subjected to starvation[19] and in several reports of starvation edema from various parts of the world.[20]

It is of some interest that the data, chemical as well as cytologic, suggest a return of the liver toward "normal" as the animal becomes severely depleted. The animal apparently attempts to conserve liver tissue after the first primary loss and as body weight falls the liver weight finally regains its original relation to body weight. Fat is lost from the liver, and the composition with regard to the constituents determined in this study becomes nearly normal. Histologically this loss of liver fat is also suggested, as well as an increase in the density of the cytoplasm and an approach toward normal of the appearance of the nuclei, cell size, and distribution of the basophilic material in the cytoplasm. It is, of course, not possible to deduce from these data that the liver has improved functionally.

SUMMARY

The progressive changes in liver composition, bromsulfalein clearance, blood and plasma volumes, thiocyanate space, total plasma nitrogen, and liver cytology have been followed in rats subjected to severe protein depletion. The effect of low and large supplies of choline upon those changes has been studied.

In agreement with the findings of previous investigators, the liver shows a very rapid loss of nitrogen, while fat and glycogen accumulate. The fat accumulation is only partially preventable with choline in the amounts used. Cytologically the livers show a rapid loss of basophilic material, the cytoplasm becomes less dense, and the sinusoids are largely obliterated. These changes are progressive for approximately one month. After more prolonged depletion the liver size approaches its original relation to body weight, the amount of fat and glycogen decrease, and the liver approaches the original cytologic state.

The fall in plasma volume and thiocyanate space during the first month of depletion was approximately proportional to the decrease in body weight. After prolonged depletion the volume occupied by these fluids per unit body weight increased slightly and proportionately. The total circulating plasma nitrogen

per 100 grams of body weight was also essentially constant for approximately one month and then increased slightly.

Although the bromsulfalein clearance per rat decreased markedly during protein depletion, this change was accounted for by the loss in weight which occurred. No proof of malfunction of the remaining liver tissue was obtained by this test.

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BROMSULFALEIN CLEARANCE

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THE conventional bromsulfalein test has been widely recognized as one of the better determinations of liver function,[6, 9] although it does not detect minor degrees of impairment. Furthermore, it has been noted that in some instances the results of this method do not coincide either with clinical evaluation or with other tests. It was hoped that by determining the rate of clearance of bromsulfalein from the blood stream rather than by measuring retention alone a more sensitive index of liver function could be obtained.

This report deals with an improved bromsulfalein test and an evaluation of liver damage in patients with chronic gall bladder disease as measured by this test.

METHOD

The subjects used in the study consisted of a control group of forty-two patients with no known liver pathology submitted to elective herniorrhaphy; another group of thirty-eight patients submitted to elective cholecystectomy; and a third miscellaneous group of eighteen subjects with known severe liver damage, including patients with hepatitis, cirrhosis, and malignant metastases to the liver.

The standard 5 per cent bromsulfalein solution was used, and all tests were run by multiple determinations with the patients under basal conditions. For this purpose an indwelling needle, attached to a glass adapter and a rubber cap,[7] was inserted into an arm vein and left in place. A sample of blood was drawn as a control before the injection of the dye, and others were obtained at intervals during the test. Small amounts of diluted heparin solution (1,000 I.U. per cubic centimeter diluted to 1 in 50 with normal saline) were injected through the rubber cap between samples to keep the needle open. The dye was administered intravenously into the other arm at a slow rate, in order to prevent the irritating effect of the concentrated material from producing a phlebitis, or, if extravascular, a severe cellulitis. Care was also necessary in the sampling to prevent hemolysis. All samples were immediately centrifuged without anticoagulant, with a portable centrifuge.

The serum was treated by a procedure modified for use with the Coleman Junior spectrophotometer Model 6-A.* One-half cubic centimeter of serum was mixed with 2 c.c. of 1 per cent saline and the mixture was divided into two equal parts. One portion was acidified by the addition of 0.05 c.c. of acidified 1 per cent HCl made by adding 2 c.c. of concentrated HCl to 100 c.c. of 1 per cent NaCl. The other portion was alkalinized by the addition of 0.05 c.c. of 5 per cent NaOH.

For each blood sample, including the control, the spectrophotometer was set at 100 (no dye) at wave length 580, with the acidified portion; then the alkalinized portion was read. In this way a correction was made for any hemolysis in each specimen and for any substances in the control serum other than bromsulfalein which might affect the reading.

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*Originally the readings were made on a photolometer, but later the more accurate spectrophotometer was used exclusively. The block comparator method of estimating retention was found too gross.

Spectrophotometric readings were converted to plasma concentration in milligrams per cent from a scale made previously with known dilutions of the dye.

With regard to dosage, subjects were divided into three groups, multiple samples being obtained in all. In one, single tests were performed; in another, different doses of dye were used on the same individual; and in the third, repeated tests were run on the same patient before and after surgery, using the same quantity in each instance. The dose used in this last group was determined preoperatively by giving variable amounts on successive days, in order to obtain a dose which would give levels high enough to be read accurately.

All results were graphed on semilogarithmic paper, using the abscissa for time and the ordinate for dye concentration. In this way the rate of removal of the dye was readily visualized. The clearance coefficient,* which represents the slope of such a graph, was used to express a numerical result of the test. The coefficients used in this study were for the ten-minute period from twenty to thirty minutes following injection of dye. The reasons for using this period are given in the discussion.

RESULTS

Types of Clearance Obtained With Multiple Samples.—Twenty reliable tests were obtained by this method. With a normal circulation time, there is a rapid rise in concentration to a peak at one to three minutes after injection, because of mixing (Fig. 1). Thereafter there is a rapid fall which, in patients with good liver function, may continue until disappearance of the dye. In the presence of poor liver function, there is a slowing in the rate of removal starting at approximately fifteen to twenty minutes and continuing until all the dye has disappeared (Fig. 2).

Normal Value for Clearance Coefficient.—It was found that there was great variation in the values of clearance coefficient in the different groups. No detectable change in plasma dye level in the ten-minute period from twenty to thirty minutes following dye injection gives a coefficient of zero. This result is obtained in cases of obstructive jaundice and the test is therefore not used in them. The group of patients with known liver damage all showed very slow excretion and coefficients less than 0.037. The gall bladder group showed better liver function; average coefficient for this group was 0.063. But a few in this group had severe liver damage, as shown by the lowest reading obtained (Table I), 0.003. The control group (hernias) had repeated preoperative tests. A clearance coefficient of 0.037 was established on the controls as the lower limit of normal (90 per cent of the group had values higher than this). Because the average for this group was 0.082, values ranging from 0.08 to 0.03 should be considered as border line and below 0.03 as definite evidence of liver damage.

Effect of Variation in Dosage.—This group of tests was made in order to determine the role of dosage in the rate of clearance in the same patient. Altogether twenty-five reliable comparisons were obtained in this group, which

*The clearance coefficient for any time interval during dye removal was approximated by the following formula:

$$k = \frac{\log_e [B_1] - \log_e [B_2]}{t_2 - t_1}$$

where k = the first approximation of the clearance coefficient, B_1 = bromsulfalein concentration at (t_1) time of collecting first sample, and B_2 = bromsulfalein concentration at (t_2) time of collecting second sample.

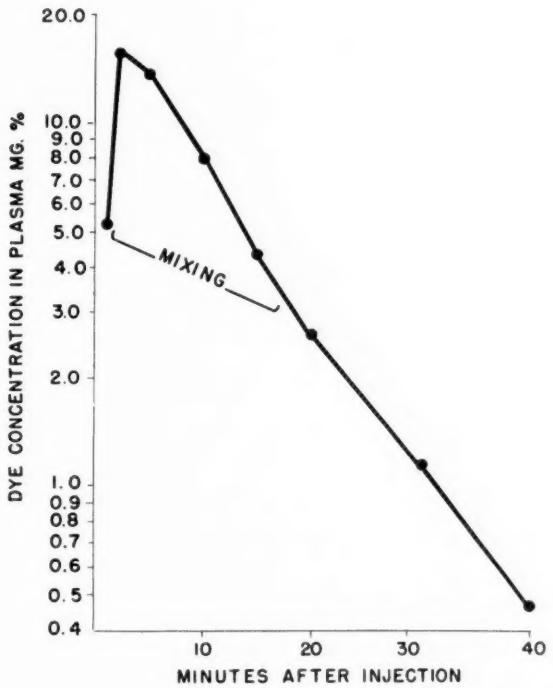

Fig. 1.—Bromsulfalein clearance in patient with good liver function. Mixing shown in early part of graph. (All graphs on semilogarithmic paper.)

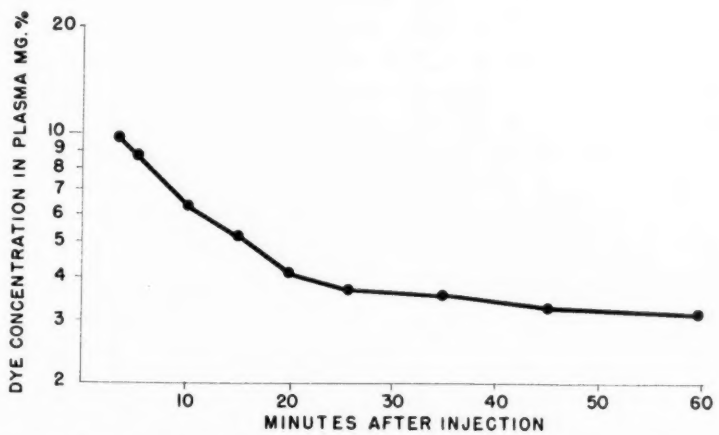

Fig. 2.—Bromsulfalein clearance in patient with poor liver function. Slow, prolonged excretion of dye.

included patients with normal and abnormal liver function. These tests were obtained under basal conditions and at such times in the clinical course that significant changes in liver function would not be expected. In most cases a 5 mg. per kilogram dose was given first; then, depending on the concentrations attained, the next dose was increased or decreased.

TABLE I. RESULTS OF PREOPERATIVE BROMSULFALEIN TEST EXPRESSED AS CLEARANCE COEFFICIENT IN PERIOD FROM 20 TO 30 MINUTES AFTER INJECTION

OPERATION	NUMBER OF CASES	RANGE	AVERAGE
Cholecystectomy	38	0.140-0.003	0.063
Herniorrhaphy	42	0.168-0.022	0.082
		Difference	0.019*

*Difference is statistically significant.

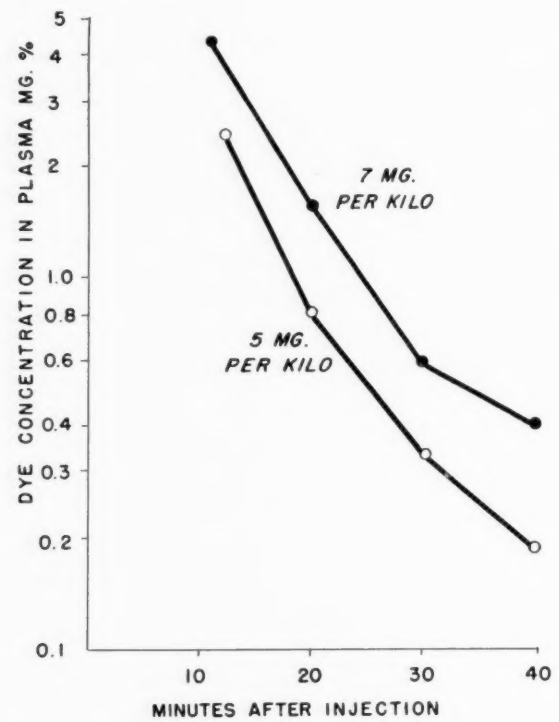

Fig. 3.—Patient with good liver function. Same clearance in spite of change in dye dose on consecutive days.

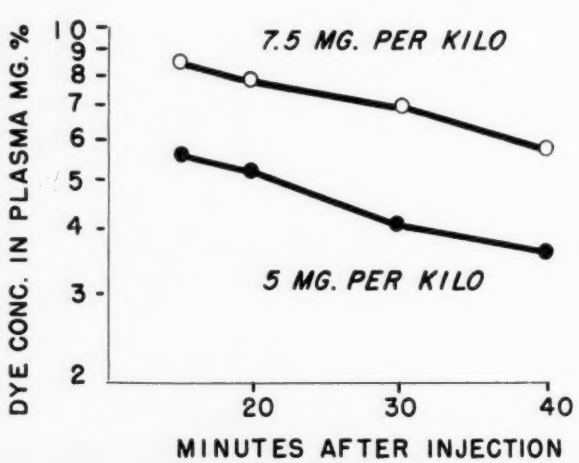

Fig. 4.—Patient with cirrhosis. Identical clearance in spite of change in dye dose on consecutive days.

Figs. 3 and 4 illustrate the rate of clearance in two patients following different dosage given on different days. The concentration of dye following a dose of 7 or 7.5 mg. was greater than that following a 5 mg. dose, but the rate of clearance was essentially the same. This is true in the patient with cirrhosis (Fig. 4), as well as in the one with a normal liver (Fig. 3). This parallelism in the rate of excretion was noted in all such tests conducted on other patients.

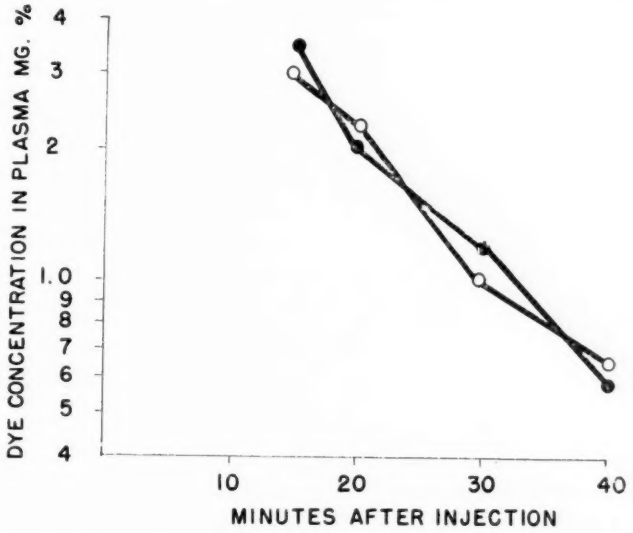

Fig. 5.—Duplication of excretion graph. Same dye dose on consecutive days.

Changes in the Clearance Coefficient Produced by Operative Load.—In patients on a fixed regimen and in a stable physiologic state, repetition of the test on consecutive days revealed identical curves for rate of clearance (Fig. 5). However, following herniorrhaphy or cholecystectomy (under gas-ether anesthesia), changes were observed. In eight individuals the rate of clearance was followed preoperatively and every day after the surgical procedure, until the preoperative clearance was re-established. In most instances a test also was done on the afternoon of the day of surgery.

Following operation in every instance there was a lowering of the clearance coefficient. The greatest depression of clearance appeared on the afternoon of the day of surgery. The return to normal in most instances took four to five days. High fever, wound hematomata, and other complications were immediately reflected by a slowing in the rate of clearance of the dye. The patients showing the best clearance before surgery demonstrated the least slowing as a result of the operation and recovered earliest.

Value of Clearance Coefficient in Determining the Minimal Liver Damage in Patients With Chronic Cholecystitis.—The results of the clearance coefficient of bromsulfalein in patients admitted for elective cholecystectomy were compared with those obtained from a group of normal subjects (herniorrhaphy). This was done to determine if the test would demonstrate liver damage in patients with gall bladder disease. As shown in Table I, the gall bladder group had a

lower coefficient of clearance as compared with the control group. The difference is statistically significant ($P = 0.015$). Since the gall bladder group consisted of older individuals than the control group, it was thought desirable to determine whether the difference between the two was not merely due to age.

TABLE II. AGE DISTRIBUTION

OPERATION	CASES	AVERAGE AGE	UNDER 40	OVER 40
Cholecystectomy	38	42.63	13 (34.21%)	25 (65.79%)
Herniorrhaphy	42	36.46	26 (61.90%)	16 (38.10%)

It can be seen from Tables II and III that when grouped according to age alone no difference was apparent, but that a difference still existed between the gall bladder and the control groups. The control patients under 40 years of age revealed a higher coefficient than those in the same age group with gall bladder disease; the same is true for those over 40.

TABLE III. RELATIONSHIP OF CLEARANCE COEFFICIENT OF BROMSULFALEIN TO AGE (RESULTS ARE THE AVERAGE SCORE FOR THE GROUP)

OPERATION	UNDER 40	OVER 40	ALL AGES
Cholecystectomy	0.059	0.066	0.063
Herniorrhaphy	0.080	0.085	0.082

DISCUSSION

The customary method of performing the bromsulfalein test is dependent upon the assumptions that the patient's blood volume is 10 per cent of the body weight and that furthermore plasma volume is proportional to body weight. It has been considered that patients receiving the same dose of dye per kilogram body weight have the same plasma concentration of dye at the start of the test. This assumption appears to be a basic source of inaccuracy. It is obvious that, in persons of asthenic habitus or with changing fluid balance because of disease or other cause, plasma volume may be far from constant with relation to body weight. Patients with cirrhosis of the liver best typify this error. A large part of their body weight may be ascitic fluid and in them the dose of dye will be abnormally great per unit of plasma volume. Because of such observations, this work was designed to investigate the manner of removal of the dye from the blood and, if possible, to find a better way of measuring its removal.

As noted earlier, in patients with poor liver function, a flattening in the slope of dye excretion was noted fifteen to twenty minutes after injection. This phenomenon has been referred to by others and has led to speculation as to its meaning.[11, 13] The liver is the main site of removal of this dye from the blood,[3, 13] while other tissues account for small amounts, especially the extrahepatic reticuloendothelial system including the spleen.[5, 8] Rosenthal, some years ago, demonstrated that the dye was adsorbed to protein and suggested that the size of this combination molecule accounted for its nonappearance in the urine of normal patients. After certain in vitro experiments,[12] he also proposed that the action of bile salts on surface tension allowed liberation of the dye from plasma protein in the liver. More recent work of Brauer and Pessotti[2] is in line with Rosenthal's theory and assigns to the true albumin fraction of human plasma the role

of binding bromsulfalein. The latter workers propose an exchange of protein-bound dye between plasma and hepatic cells as the basic mechanism. This theory of removal from plasma seems acceptable. Retention of dye following India ink injection[4, 8, 11] may be just a mechanical blocking of the Kupffer cells with resulting decreased surface area for this physical reaction to occur.

With the usual dose of 2 or 5 mg. per kilogram of body weight, the blood is cleared of the dye in from thirty to forty-five minutes,[10] but it keeps appearing in the bile for at least two hours and in some instances for as long as five or six hours.[3, 13] Excretion from the liver into bile is therefore obviously a separate mechanism. In patients with liver disease, appearance in the bile is delayed and disappearance from the bile is slow. It seems reasonable then to assume that, in these cases, slow removal from the blood after fifteen or twenty minutes is a reflection of excretion from the liver into the bile. This is in contrast to the first fifteen or twenty minutes, which measures mainly transfer from plasma to liver, and so represents only one phase of the mechanism. This assumption would explain the fact that in nearly every patient, normal or abnormal, early fall in blood levels is relatively rapid, but the slow removal in liver disease is the most noticeable in the later stage of the test. If the liver can dispose of the dye rapidly, it may continue to remove it from the blood at its maximum rate of acquisition, and so in the normal subject no flattening of the graph occurs. However, if excretion into the bile is slow, then the liver becomes blocked and hence can lower the blood level later only at this reduced rate of clearance into the bile. This biphasic mechanism, then, is one reason why the rate of excretion after twenty minutes can be considered more indicative of actual liver function than the response before this time.

The choice of thirty minutes as the late limit of the determinations is based on the principle of decreasing reliability of readings with high dilutions. At forty minutes there is often little or no dye left with the 5 mg. dose. Therefore, accurately timed samples at twenty and thirty minutes have been taken and the clearance coefficient obtained for this time interval has been considered as an index of the efficiency of the removal mechanism (mainly liver). If greater accuracy were required, three samples could be taken at twenty, twenty-five, and thirty minutes after injection, the coefficient from twenty to twenty-five and from twenty-five to thirty minutes calculated, and the average determined. Thus, without prolonging the test, any error in one specimen could be minimized.

It may not be amiss to call attention to the work of Blumberg and Schloss[1] who have shown that circulatory inadequacy will slow dye excretion. Thus, poor clearance in a cardiac patient with congestive failure may be evidence of poor circulation alone or combined with liver damage.

SUMMARY AND CONCLUSIONS

The results obtained with a modification of the bromsulfalein test, utilizing the clearance coefficient, are presented. For practical purposes, the coefficient during the interval from twenty to thirty minutes after dye injection was found to be most useful, a lower limit of normal for this period being determined.

This procedure measures the actual manner of removal of the dye and is a better index of liver function than present methods of studying dye retention. With it a comparison was made between a group of subjects with normal liver function and patients who were candidates for elective cholecystectomy and hence expected to show liver damage, and a significant difference was demonstrated between the two types of subjects.

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THE CONCENTRATION OF COMPONENT A IN BLOOD, ITS ASSAY AND RELATION TO THE LABILE FACTOR

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FOR clinical purposes it is advantageous to divide the clotting factors required for the formation of thrombin into two categories: the thromboplastin formers, and constituents of the prothrombin complex.[1] The latter are essential for prothrombin activity as measured by the one-stage prothrombin time. According to Quick and associates, the agents in the complex are: the labile factor, components A and B, and bound calcium.[2, 3, 5] The labile factor is characterized by its instability, especially in decalcified plasma, and its nonadsorbability by tricalcium phosphate. Component A is the principle which is adsorbable by tricalcium phosphate, requires vitamin K for its synthesis, and is deficient in one type of congenital hypoprothrombinemia. It is probably this component which is generally called prothrombin by other investigators. Component B is difficult to define. Its existence is postulated to explain a second type of congenital hypoprothrombinemia, in which the labile factor and component A appear to be normal.[4] This type of hypoprothrombinemia is particularly important because it is hereditary and the lowered fixed prothrombin time may run through several generations. Little is known concerning the functions of calcium in the formation of thrombin, but recent studies clearly show that it is not ionic but bound calcium that participates in the reaction.[5]

The desirability of having methods for the quantitative estimation of the various constituents of the prothrombin complex is obvious. Recently we developed a simple procedure to estimate the concentration of the labile factor and found that rabbit blood contains fifty times and dog blood ten times as much as human blood.[6] In the present report experiments are described which not only yield a procedure for the estimation of component A, but also furnish results which show its relationship to the labile factor.

METHODS AND MATERIAL

The one-stage method for the prothrombin time procedure was employed exactly as outlined by its originator.[7] The thromboplastin used was acetone-dehydrated rabbit brain. It uniformly yielded a prothrombin time of 11 to 12 seconds for normal human plasma.

The tricalcium phosphate treated plasma was prepared according to directions previously described.[6] Plasma thus prepared is free of component A, but retains practically all of its fibrinogen and all of the labile factor. Care was taken to take only the minimum quantity of tricalcium phosphate required, i.e., an amount just sufficient to render the plasma incoagulable to excess thromboplastin and an optimum concentration of calcium.

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Each lot of stock solution was tested on the oxalated plasmas of the various species employed in the study. All studies were carried out on oxalated plasma (1 vol. of 0.1M sodium oxalate to 9 vol. of blood). It should be emphasized that tricalcium phosphate does not remove component A from citrated plasma.[5] For convenience, plasma treated with tricalcium phosphate is named in this paper either "calcium phosphate plasma" or "$Ca_3(PO_4)_2$ plasma."

Isolation of a Concentrate of Component A.—The tricalcium phosphate after its removal from the plasma by centrifugation was washed with water and again packed by centrifuging. The water was poured off and the last traces adhering to the sides of the tube were removed with a filter paper. For every cubic centimeter of plasma treated, 0.1 c.c. of 0.2M sodium citrate was added and the mixture stirred for ten minutes. The tricalcium phosphate was removed by centrifugation. The eluate contained approximately 90 per cent or more of the component A present in the plasma.

Vitamin K Deficiency.—Newly hatched chicks were put on a vitamin K free diet and the procedure was followed as recently outlined.[9]

RESULTS

The Estimation of the Concentration of Component A in Plasma by Mixing With Plasma Devoid of This Factor.—Since complete avitaminosis K can be achieved readily in chicks, a plasma showing little or no prothrombin activity was easily obtained. When such a plasma was progressively diluted with normal hen plasma, the prothrombin time decreased in accordance with the typical hyperbolic curve until the 50 per cent dilution was reached. After this the prothrombin time remained the same as that of the normal hen plasma (Table I).

TABLE I. THE EFFECT ON THE PROTHROMBIN TIME WHEN AVITAMINOTIC K CHICK PLASMA AND HEN PLASMA TREATED WITH TRICALCIUM PHOSPHATE ARE PROGRESSIVELY DILUTED WITH NORMAL HEN PLASMA

Plasma of vitamin K deficient chick (c.c.)	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01	0.0
Normal hen plasma (c.c.)	0.0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.1
Prothrombin time (sec.)	∞	16	14	13½	12	11½	11	11	11	11	11
Hen plasma treated with $Ca_3(PO_4)_2$ (c.c.)	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01	0.0
Normal hen plasma (c.c.)	0.0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.1
Prothrombin time (sec.)	∞	17	15	14	12	11½	11	11	11	11	11

Exactly similar results were obtained when normal hen plasma was diluted with hen plasma from which component A was adsorbed with tricalcium phosphate. These results clearly show that the concentration of component A in normal hen plasma can be reduced almost 50 per cent before the prothrombin time begins to increase. In other words component A may vary between 50 and 100 per cent without affecting the prothrombin time. This was strikingly illustrated by injecting vitamin K_1 into a chick with an initial prothrombin time of 10 minutes. Four hours later it was reduced to 11 seconds, i.e., to the same level as that of a hen on a complete diet. However, on comparing the prothrombin times obtained when this and normal hen plasma were mixed with plasma free of component A, it is plain that the concentration of component A in the chick blood was definitely lower (Table II). If one uses the data of Table I as a guide, it can be concluded that 0.03 c.c. of hen plasma contains as much component A

as 0.05 c.c. of the chick plasma, yet both had a normal prothrombin time. It is clear that component A does not become the determinant of the prothrombin time until its concentration is reduced below 50 per cent of the normal level.

TABLE II. THE CONCENTRATION OF COMPONENT A IN THE BLOOD OF A VITAMIN K DEFICIENT CHICK AFTER THE ADMINISTRATION OF VITAMIN K_1

Prothrombin time of chick plasma before giving vitamin K_1		10 min.	
Prothrombin time of chick plasma 4 hr. after giving 10 γ of vitamin K_1		11 sec.	
Vitamin K deficient chick plasma	0.05 c.c.	Vitamin K deficient chick plasma	0.05 c.c.
Normal hen plasma	0.05 c.c.	Chick plasma after vitamin K	0.05 c.c.
Prothrombin time (sec.)	11½	Prothrombin time (sec.)	13½
Hen plasma treated with $Ca_3(PO_4)_2$	0.05 c.c.	Hen plasma treated with $Ca_3(PO_4)_2$	0.05 c.c.
Normal hen plasma	0.05 c.c.	Chick plasma after vitamin K_1	0.05 c.c.
Prothrombin time (sec.)	11½	Prothrombin time (sec.)	13½
Normal hen plasma	0.05 c.c.	Chick plasma after vitamin K_1	0.05 c.c.
Saline	0.05 c.c.	Saline	0.05 c.c.
Prothrombin time (sec.)	14½	Prothrombin time (sec.)	15

Because of the difficulty of attaining severe vitamin K deficiency hypoprothrombinemia in mammals and the relative infrequency of avitaminosis K in man, it was not possible to run a dilution titration such as was made with chick plasma. One of us (A. J. Q.), however, found in his records of 1938 two important experiments. The first was the determination of the effect of mixing the plasma of a newborn infant having a prothrombin time of 45 seconds with an equal volume of normal human plasma. A prothrombin time of 12 seconds was obtained. Similarly, when the plasma of a jaundiced patient (prothrombin time 48 seconds) was mixed with normal human plasma, the resulting prothrombin time was 12½ seconds. In both plasmas component A was greatly diminished, yet on adding half the volume of normal plasma the prothrombin time was restored to normal even though the mixture contained only about one-half as much component A as normal plasma.

By using fresh human plasma treated with tricalcium phosphate as diluent, the concentration of component A in normal plasma can be determined (Table III). Interestingly, almost identical results were obtained when rabbit plasma adsorbed with calcium phosphate was employed as diluent, provided the human plasma was collected with the utmost care to preserve platelets. It will be observed that as the calcium phosphate plasma was mixed with increasing amounts of normal plasma, the prothrombin time decreased until a dilution of 50 per cent was attained. Beyond this the prothrombin time remained the same as that of normal plasma. As in the case of hen plasma, the prothrombin time remained normal until the concentration of component A was reduced below 50 per cent of the amount present in normal blood. Again it is clear that the concentration of component A is not the determinant of the prothrombin time. The labile factor likewise is not the deciding factor. Rabbit plasma contains fifty times as much labile factor as human plasma, yet the results with rabbit and human calcium phosphate treated plasmas as diluents are almost identical.

TABLE III. THE EFFECT ON THE PROTHROMBIN TIME WHEN NORMAL HUMAN PLASMA IS PROGRESSIVELY DILUTED WITH HUMAN AND WITH RABBIT PLASMA FROM WHICH COMPONENT A WAS REMOVED BY ADSORPTION WITH TRICALCIUM PHOSPHATE

Normal human plasma (c.c.)*	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01	0.00
Normal human $Ca_3(PO_4)_2$ plasma (c.c.)	0.0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.10
Prothrombin time (sec.)	12	12	12	12	12	12	13	15	18	26	∞
Normal human plasma (c.c.)*	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01	0.00
Rabbit $Ca_3(PO_4)_2$ plasma (c.c.)	0.0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.10
Prothrombin time (sec.)	12	11½	11½	11½	11½	12	13	15	18	40	∞

*The blood was collected in a silicone-coated syringe and transferred to a silicone-coated test tube containing sodium oxalate.

Congenital Hypoprothrombinemia. Deficiency of Component A.—It was fortunate that in the pursuit of this problem two patients with congenital hypoprothrombinemia of the type that lacks component A were available. Studies of the blood of these two brothers were reported previously.[4] Characteristically, when 1 volume of their plasma was mixed with an equal volume of normal human plasma, a normal prothrombin time was attained. In fact when the hypoprothrombinemic plasma was progressively diluted with normal plasma, a curve was obtained (Table IV) which was almost identical with that observed when avitaminotic K chick plasma was diluted with normal hen plasma. This is easily explained by postulating that both the chick's and the patient's plasma lacked component A, and since normal plasma contains nearly twice as much as is needed to produce the normal fixed prothrombin time, this minimum value is obtained when 50 per cent of the volume of normal plasma is added to the defective plasma. Interestingly, when the deficient plasma is diluted with normal human plasma treated with tricalcium phosphate, the prothrombin time increases progressively with the dilution and is practically identical with the results obtained when saline is the diluent. When rabbit plasma treated with tricalcium phosphate is used, the prothrombin times again increase progressively, but the values are shorter than those obtained with human calcium phosphate plasma. An explanation for this finding requires further study. It is clear that the plasmas of the two boys which have a consistent prothrombin time of 19 to 21 seconds lack component A. According to Quick's original prothrombin curve, those values indicate a prothrombin activity of 25 to 30 per cent of normal. In the light of the present findings, the concentration of component A in terms of per cent of the concentration of A in normal plasma may actually be lower than 25 per cent.

The present findings as well as other results from recent studies carried out in our laboratory show that the prothrombin time as determined by the one-stage procedure does not measure any particular constituent of the prothrombin complex but rather their composite effect. The true determinant of the relatively fixed prothrombin time of 12 seconds in normal human plasma still remains unexplained. Because of the low concentration of the labile factor, after twenty-

TABLE IV. THE EFFECT OF THE PROTHROMBIN TIME WHEN PLASMA FROM A PATIENT WITH CONGENITAL HYPOTHROMBINEMIA (DEFICIENCY OF COMPONENT A) IS DILUTED WITH HUMAN AND WITH RABBIT PLASMA ADSORBED WITH $Ca_3(PO_4)_2$ AND WITH SALINE

Plasma from patient (c.c.)*	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01
Normal human plasma (c.c.)	0.0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
Prothrombin time (sec.)	21	14	13	12	11½	11	11	11	11	11
Plasma from patient (c.c.)*	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01
Human $Ca_3(PO_4)_2$ plasma (c.c.)	0.0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
Prothrombin time (sec.)	21	23	24	25½	27	38	55	70	29	600
Plasma from patient (c.c.)*	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01
Rabbit $Ca_3(PO_4)_2$ plasma (c.c.)	0.0	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
Prothrombin time (sec.)	21	19½	19½	20½	21	22	23½	24½	35	53
Plasma from patient (c.c.)*	0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03		
Saline solution	0.0	0.01	0.02	0.03	0.04	0.05	0.06	0.07		
Prothrombin time (sec.)	21	24	25	27	31	39	50	75		

*The patient was a 4-year-old boy. He and his brother, age 7½ years, had prothrombin times which ranged between 19 and 22 seconds. Both had a mild bleeding tendency.

four hour storage human plasma may show a decrease of 50 per cent of prothrombin activity caused by destruction of this agent, whereas rabbit plasma may show no loss, as determined by the prothrombin time, for a week or longer because the labile factor is in great excess. Component A may be decreased 50 per cent, according to the result of this study, before the prothrombin time is prolonged. These findings do not condemn the one-stage method, but rather emphasize that it measures the over-all prothrombin activity. For the determination of the specific factors, methods must be devised which are basically modifications of the one-stage procedure. Thus, a simple method of the assay of the labile factor was developed in our laboratory in which stored human plasma is the testing medium.[6] From the results of the present study, a simple determination of component A can be outlined. A sample of fresh oxalated plasma to be tested is mixed with increasing volumes of fresh normal human plasma treated with tricalcium phosphate, and the prothrombin times of the mixtures are determined. If the concentration of component A is normal, the results will be identical with those given in Table III. By means of eluting with sodium citrate the tricalcium phosphate to which component A is adsorbed, a new means for assaying this component becomes available as will now be described.

The Concentration of Component A in Human and in Dog Plasma as Determined by Re-adding to Component A Free Plasma the Factor Concentrated by Adsorption and Elution.—In this study evidence was offered to show that tricalcium phosphate removes only component A from oxalated plasma; therefore plasma thus treated becomes an ideal assay medium for determining component A. All that is required is to add it to the calcium phosphate plasma and determine the prothrombin time. Since the only significant component adsorbed is A and since sodium citrate elutes it almost completely, the eluate is presumably a relatively pure and concentrated solution of component A. Since 0.1 c.c. of sodium citrate is employed to elute the product adsorbed from 1 c.c. of plasma, this amount when re-added to 1 c.c. of calcium phosphate treated plasma should restore the prothrombin time to normal. This was found to be true if human

eluate was added to human plasma, and dog eluate to its respective plasma, but when human eluate was added to dog plasma ($Ca_3(PO_4)_2$ treated) or rabbit plasma somewhat shorter times were obtained. On the other hand, when dog eluate was added to human plasma, the resulting prothrombin time was approximately the same as that of normal human plasma. In Table V the prothrombin times obtained by adding component A eluate in progressively decreasing amounts to deprothrombinized plasma are presented. It will be observed that by adding decreasing amounts of human eluate to human deprothrombinized

TABLE V. THE QUANTITATIVE EFFECT ON THE PROTHROMBIN TIME OF ADDING COMPONENT A (CONCENTRATED BY ADSORPTION AND ELUTION) TO HUMAN, DOG, AND RABBIT PLASMAS TREATED WITH $Ca_3(PO_4)_2$

Normal human $Ca_3(PO_4)_2$ plasma (c.c.)	0.09	0.091	0.092	0.093	0.094	0.095	0.096	0.097	0.098	0.099
Eluate from normal human plasma (c.c.)	0.01	0.009	0.008	0.007	0.006	0.005	0.004	0.003	0.002	0.001
Prothrombin time (sec.)	12	12	12½	13½	14	15	17	21	28	45
Expected value from Quick's prothrombin curve	12	12	12½	13½	14	15	17	19	25	38
Rabbit $Ca_3(PO_4)_2$ plasma (c.c.)	0.09	0.091	0.092	0.093	0.094	0.095	0.096	0.097	0.098	0.099
Eluate from normal human plasma (c.c.)	0.01	0.009	0.008	0.007	0.006	0.005	0.004	0.003	0.002	0.001
Prothrombin time (sec.)	10½	10½	11	11	11½	12	13¼	14	19	31
Normal human $Ca_3(PO_4)_2$ plasma (c.c.)	0.09	0.091	0.092	0.093	0.094	0.095	0.096	0.097	0.098	0.099
Eluate from dog plasma (c.c.)	0.01	0.009	0.008	0.007	0.006	0.005	0.004	0.003	0.002	0.001
Prothrombin time (sec.)	10	10	10½	11	11¼	11½	12	12	12½	19
Dog $Ca_3(PO_4)_2$ plasma (c.c.)	0.09	0.091	0.092	0.093	0.094	0.095	0.096	0.097	0.098	0.099
Eluate from dog plasma (c.c.)	0.01	0.009	0.008	0.007	0.006	0.005	0.004	0.003	0.002	0.001
Prothrombin time (sec.)	6	6	6	6	6	6	6¼	6¾	7	8½

plasma, prothrombin time values are obtained which correspond closely to the values of Quick's prothrombin curve. It also should be noted that only one-third to one-fifth as much dog eluate as human eluate must be added to human deprothrombinized plasma to obtain a normal prothrombin time of 12 seconds. This clearly shows that dog plasma contains several times as much component A as is normally present in human plasma. This agrees with the original findings obtained with the one-stage method that dog plasma contains five times as much prothrombin as human plasma[10] and casts serious doubt on the hypothesis that the difference between the prothrombin times of dog and human plasma is due to variations in conversion factors.

The reason dog eluate added to human deprothrombinized plasma produces a prothrombin time of only 10 seconds instead of 6 seconds as in dog plasma is the inadequate concentration of the labile factor in human blood. Either component A or the labile factor can be the limiting factor in the production of thrombin and in determining the prothrombin time. This is strikingly illustrated by Experiment I. A small quantity of rabbit plasma supplied sufficient labile factor to reduce the prothrombin time to 10 seconds. This emphasizes the importance of controlling the concentration of the labile factor in assaying component A. Fortunately, the concentration of the labile factor has been found to have a fixed and relatively constant level for any particular species. When human plasma is employed as the assaying medium, it should be used within a few hours after the blood is collected and be preserved in an ice bath. Normal human plasma does not contain sufficient labile factor to permit its use for assaying concentrated solutions of eluate such as is obtained from dog plasma.

EXPERIMENT I

Stored human $Ca_3(PO_4)_2$ plasma	0.09 c.c.	Fresh human $Ca_3(PO_4)_2$ plasma	0.09 c.c.
Eluate from human plasma	0.01 c.c.	Eluate from human plasma	0.01 c.c.
Prothrombin time	25 sec.	Prothrombin time	12 sec.

Stored human $Ca_3(PO_4)_2$ plasma	0.08 c.c.
Rabbit $Ca_3(PO_4)_2$ plasma	0.01 c.c.
Eluate from human plasma	0.01 c.c.
Prothrombin time	10 sec.

Prothrombin time of stored plasma 25 sec.

The Concentration of Component A in the Plasma of Congenital Hypoprothrombinemia.—The lack of component A in the type of congenital hypoprothrombinemia studied is clearly shown by the results given in Experiment II. The prolonged prothrombin time of Subject B. B. is due purely to a deficiency of component A. The eluate obtained from his plasma when tested for component A potency either on normal calcium phosphate plasma or on his own plasma yielded the same low result, whereas the eluate from normal human plasma restored his prothrombin level to normal exactly as it did in normal calcium phosphate plasma.

EXPERIMENT II

Subject B.B.: Prothrombin time	20 sec.	(Congenital hypoprothrombinemia)	
Normal subject: Prothrombin time	11½ sec.		
B.B. $Ca_3(PO_4)_2$ plasma	0.09 c.c.	B.B. $Ca_3(PO_4)_2$ plasma	0.09 c.c.
Eluate from B.B. plasma	0.01 c.c.	Eluate from normal human plasma	0.01 c.c.
Prothrombin time	29 sec.	Prothrombin time	11½ sec.
Normal human $(Ca_3(PO_4)_2$ plasma	0.09 c.c.	Normal human $Ca_3(PO_4)_2$ plasma	0.09 c.c.
Eluate from B.B. plasma	0.01 c.c.	Eluate from normal human plasma	0.01 c.c.
Prothrombin time	25 sec.	Prothrombin time	11½ sec.

The Concentration of Component A in Avitaminosis K.—After chicks were on a vitamin K free diet for ten days, a severe hypoprothrombinemia was produced, in fact, a prothrombin time greater than 8 minutes occurred in about one-fourth of a group of twenty chicks. An eluate of the plasma of these chicks

showed no prothrombin potency when tested on hen calcium phosphate plasma, indicating a complete lack of component A. An eluate obtained from normal hen plasma when tested on the plasma of these avitaminotic K chicks immediately restored the prothrombin to the same level as this amount of eluate did in normal hen plasma treated with tricalcium phosphate, Experiment III. It is logical to conclude that deficiency of vitamin K produces a pure component A type of hypoprothrombinemia.

EXPERIMENT III

Prothrombin time of normal hen plasma			11 sec
Prothrombin time of vitamin K deficient Chick 1			480 sec.
Prothrombin time of vitamin K deficient Chick 2			600 sec.
Prothrombin time of vitamin K deficient Chick 3			Infinity
Hen $Ca_3(PO_4)_2$ plasma	0.09 c.c.	Chick 1 plasma*	0.09 c.c.
Eluate from hen plasma	0.01 c.c.	Eluate from hen plasma	0.01 c.c.
Prothrombin time	13 sec.	Prothrombin time	13½ sec.

*The prothrombin times of the plasmas of Chicks 2 and 3 after the addition of the eluate of normal hen plasma were 12½ and 13 seconds respectively. Previous treatment of these plasmas with tricalcium phosphate did not change their response to eluate.

Concentration of Component A in Plasma After Administration of Dicumarol.—From the work in our laboratory, evidence has been obtained that the hypoprothrombinemia caused by Dicumarol is not identical with that brought about by vitamin K deficiency. Recently Dam and Söndergaard[11] have also come to the conclusion that the two types of hypoprothrombinemia are not identical, although they emphasize that the primary deficiency in both is the "classical prothrombin."

In the present investigation, no attempt was made to determine the basic difference between the two types of hypoprothrombinemia. The sole objectives were to ascertain the quantitative effect Dicumarol had on the concentration of component A and to determine the response of Dicumarol plasma to the addition of an eluate of component A obtained from normal plasma. It was found, as shown in Table VI, that the response of Dicumarol plasma to the eluate from dog plasma was the same as that of normal plasma. This shows that the hypoprothrombinemia resulting from Dicumarol can be promptly and completely corrected by the addition of a concentrated solution of component A, i.e., the eluate from the tricalcium phosphate adsorbed plasma.

TABLE VI. THE RESPONSE OF NORMAL AND DICUMAROL PLASMA TO THE ADDING OF COMPONENT A OBTAINED AS AN ELUATE FROM NORMAL AND DICUMAROL PLASMA

Normal dog $Ca_3(PO_4)_2$ plasma (c.c.)	0.098	0.096	0.094	0.092	0.09
Eluate from normal dog plasma (c.c.)	0.002	0.004	0.006	0.008	0.01
Prothrombin time (sec.)	16	8	7	6½	6
Dicumarol dog $Ca_3(PO_4)_2$ plasma (c.c.)	0.098	0.096	0.094	0.092	0.09
Eluate from normal dog plasma (c.c.)	0.002	0.004	0.006	0.008	0.01
Prothrombin time (sec.)	16	8	7	6½	6½

Normal dog $Ca_3(PO_4)_2$ plasma (c.c.)	0.09	Dicumarol dog $Ca_3(Po_4)_2$ plasma (c.c.)	0.09
Eluate from Dicumarol plasma (c.c.).*	0.01	Eluate from Dicumarol plasma (c.c.)*	0.01
Prothrombin time (sec.)	38	Prothrombin time (sec.)	67

*Eluate obtained from Dicumarol plasma which had a prothrombin time of 48 seconds.

From the results recorded in Experiment IV it is evident that this correction can be effected not only in vitro but in vivo as well. One can readily comprehend the therapeutic implications of this experiment. By two simple procedures, namely, adsorption with tricalcium phosphate and elution with sodium citrate, one can concentrate the quantity of component A contained in 500 c.c. of blood to 25 c.c. and have it immediately suitable for intravenous injection. The clinical evaluation of the eluate is being studied at present.*

EXPERIMENT IV

Dog F., weight 8 kg., given 5 mg. of Dicumarol per kg. of body weight daily for 4 days	
Eluate (concentrated component A) prepared from 22 c.c. of fresh normal dog plasma	
Prothrombin time before injection of eluate	30 sec.
Prothrombin time after injection of eluate	16 sec.

The basic defect in Dicumarol poisoning is lack of component A, the same as in avitaminosis K. This makes the hypothesis made by one of us (A.J.Q.),[2] that Dicumarol causes a decrease of component B, untenable. Nevertheless, there is evidence that the hypoprothrombinemia due to lack of vitamin K is not identical with that due to Dicumarol. It will be noted that the eluate obtained from Dicumarol plasma is more effective in normal than in Dicumarol plasma. Studies are being carried out to determine whether this observation is significant.

SUMMARY

When normal oxalated plasma is added in progressively increasing amounts to plasma from which component A of prothrombin is absent or very low due either to vitamin K deficiency, removal by adsorption with tricalcium phosphate, or congenital lack, the prothrombin time is characteristically decreased until approximately 1 volume is added to 1 volume of the defective plasma. After this the prothrombin times become the same as that of the normal plasma. This indicates that normal plasma supplies about twice as much component A as is required to attain a normal prothrombin time and that therefore it is apparently not the specific determinant of the prothrombin time even in the presence of an excess of the labile factor.

By means of adsorbing oxalated plasma with tricalcium phosphate and treating the latter with sodium citrate, an eluate is obtained which contains almost all of the component A present in the plasma. When this eluate is added in an amount equivalent to the volume of normal plasma from which it was obtained to plasma lacking component A, the normal prothrombin time is restored. By adding progressively decreasing amounts of the eluate to component A free plasma, a series of values of prothrombin time are obtained which correspond well with the original prothrombin curve. Component A is active only if an adequate amount of the labile factor is present. The isolation of component A as an eluate and the testing of it on fresh tricalcium phosphate treated plasma offer a new method for its quantitative estimation.

*In cooperation with the Junior League Blood Center of Milwaukee.

The therapeutic possibilities of employing the eluate in various types of component A deficiencies are discussed. Its efficacy in reducing the prothrombin time in Dicumarol poisoning is illustrated in dogs.

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INTENSIVE IMMUNIZATION OF AN ALREADY SENSITIZED Rh-NEGATIVE WOMAN; BIRTH OF A MILDLY DISEASED BABY

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ONCE a woman has given birth to an Rh-positive child with a fatal form of erythroblastosis, it is very uncommon for her to give birth to one less diseased.[1, 2, 3, 4] This is still more rare when the woman has been sensitized by both pregnancy and transfusion. When the donor of the transfusion is the husband the outlook is almost hopeless. This situation developed in the family here recorded. The record was as follows.

Family Blood Groups.—

Dr. E. N. E. (husband): Group 0, MN, P positive, S negative, CDe cDe (R_1R_0).

Mrs. E. N. E. (wife): Group 0, MN, P positive, S negative, cde cde (r r).

N. J. E. (son): Group 0, S negative, CDe cde (R_1 r).

Mr. C. E. (father of Dr. E. N. E.): Group 0, S negative, CDe cde (R_1 r).

Mrs. C. E. (mother of Dr. E. N. E.): Group A_1, cDE cDe (R_2 R_0).

Mrs. N. C. (sister of Dr. E. N. E.): CDe cDe (R_1R_0).‡

Pregnancy Record of Mrs. E. N. E., white, age 35 years.—

October, 1940: Abortion at twelve weeks followed by hemorrhage and pneumonia. Transfusion of 500 c.c. of husband's blood without reaction.

May, 1942: Delivery of living child (N. J. E.), moderately jaundiced for one week.

August, 1945: Spontaneous abortion at eight weeks.

August, 1946: Delivery of macerated fetus at thirty-nine weeks. Active fetal movements ceased two weeks prior to delivery. A necropsy was performed on the fetus.§ The following are the gross findings:

> Fetus, weight 3500 gm., length 48 cm. Very marked edema over the whole body, especially occiput, buttocks and extremities. Numerous, ruptured blebs were present, leaving a raw surface. Lips and tongue were purplish black in color. There was no fluid in the pleural or pericardial cavities. The lungs were brown, firm and on section like cut meat. The heart and g.i. tract were normal. The liver was enlarged and of purplish brown color. The spleen was also enlarged.

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‡

	C	D	E	c	e	C^w
Dr. E. N. E.	+	+	−	+		−
Mrs. E. N. E.	−	−	−	+		−
N. J. E.	+	+	−	+		−
Mr. C. E.	+	+	−	+		−
Mrs. C. E.	−	+	+	+	+	−
Mrs. N. C.	+	+	−	+		−

These tests were performed by the Vancouver Red Cross Laboratory, Vancouver, B.C., Canada, and by Dr. R. R. Race, The Lister Institute, London, England.

§By Dr. R. J. D. McNeely, Pathologist, Royal Jubilee Hospital, Victoria, B. C., Canada.

TABLE I

DATE	AMOUNT OF INJECTIONS (C.C.)	Rh ANTIBODY TITERS			SUBJECTIVE REACTIONS	NOTES
		SALINE ANTI-C	ALBUMIN ANTI-C	ALBUMIN ANTI-D		
	Intravenous					
1947 Jan. 14	2	0	0	1:64	0	
Jan. 21	3				0	
Jan. 28	4.5	1:2	0	1:512	+	
Feb. 4	6				+	Benzidine test urine—trace
Feb. 11	8				+	
Feb. 18	9	1:8	0	1:130,000	0	
Feb. 26	10.5	1:8	0	1:16,000,000*	0	
Mar. 5	13	1:8	0	1:1,028	++	
Mar. 12	15	1:16	0	1:4,000	0	
Mar. 19	17.5	1:4	0	1:16,000	++	
Mar. 25	19.5	1:8	0	1:4,000	+++	
Apr. 2	23.5	1:8	0	1:2,000	++	
Apr. 8	27				+++	
Apr. 16	30	1:8	0	1:4,000	++	
Apr. 23	33	1:8	0	1:8,000	0	
May 1	37	1:8	0	1:1,000	++	
May 7	40	1:16	0	1:1,000	+	Serum albumin—4.8 Gm.
May 14	43	1:16	0	1:1,000	0	Serum globulin—3.3 Gm.
May 21	46	1:16	0	1:1,000	+	
May 29	50	1:16	0	1:512	++++	
June 4	53	1:8	0	1:512	+++	Urine benzidine—+
June 11	53	1:2	0	1:512	+++	Urine benzidine—+
June 18	54	1:2	0	1:256	++	Urine benzidine—++
June 25	56	1:2	0	1:128	++++	
July 2	50	1:2	0	1:128	++++	Icteric index—10, at height of reaction
July 9	35	1:2	0	1:256	++++	
July 16	20	1:2	0	1:256	++++	Last menstrual period (beginning of pregnancy)
July 25	0	Trace	1:8	1:128		
Aug. 22	5				++++	
	Intramuscular					
Sept. 1	5				0	
Sept. 6	8				0	
Sept. 11	8	Trace	1:16	1:128	0	
Sept. 16	8				0	
Sept. 21	8				0	
Sept. 26	8	0	0	1:128	0	
Oct. 1	8				0	
Oct. 6	8				0	
Oct. 10	8				0	
Oct. 15	8				0	
Oct. 20	8				0	
Oct. 25	8				0	
Oct. 30	8	0	0	1:128	0	
Nov. 4	8				0	
Nov. 9	8				0	
Nov. 14	8				0	
Nov. 19	8				0	
Nov. 24	8				0	
Nov. 29	8				0	
Dec. 4	8	0	0	1:128	0	
Dec. 9	8				0	

TABLE I—CONT'D

DATE	AMOUNT OF INJECTIONS (C.C.)	Rh ANTIBODY TITERS			SUBJECTIVE REACTIONS	NOTES
		SALINE ANTI-C	ALBUMIN ANTI-C	ALBUMIN ANTI-D		
Dec. 14	8				0	
Dec. 19	8				0	
					0	23rd week of pregnancy
Dec. 24	8	0	0	1:512		
Dec. 29	8				0	
1948 Jan. 3	8				0	
Jan. 8	8				0	
Jan. 13	8	0	0	1:512	0	
Jan. 18	8				0	
Jan. 23	8				0	
Jan. 28	8				0	
Feb. 2	8				0	
Feb. 7	8				0	
Feb. 12	8				0	
Feb. 17	8				0	
Feb. 22	8				0	
Feb. 27	8	0	0	1:512	0	
Mar. 3	8				0	
Mar. 8	8				0	
Mar. 13	8				0	
Mar. 18	8	0	0	1:1,000	0	
Mar. 23	8				0	
Mar. 29	0	0	0	1:4,000		Delivery at 37th week
Oct. 29		0	0	1:512		Seven months post partum

Data on Rh-isoimmunized woman (Mrs. E. N. E.) showing injections intravenously and intramuscularly of husband's Rh-positive blood. The Rh antibody titers are shown. (Tests performed by the Vancouver Red Cross Laboratory, Vancouver, British Columbia, and occasionally confirmed by Dr. Philip Levine, Ortho Research Foundation, Raritan, N. J.) Throughout the titrations an absent or weak agglutination (negative prozone), to the albumin (blocking) anti-D factor, was noted in the first two to five tubes. Subjective reactions of the patient were graded as follows:

+, Slight, transient shooting pains, usually of fingers.

++, Few chills; joint pains (see +); headache for one hour.

+++, More severe chills; pains and headache lasting for two hours.

++++, Chills; joint pains; backache; severe headache; temperature to 101°F. Reaction lasting about three hours but responding in part to antihistaminic drugs.

Tests for hemoglobin in the urine (benzidine test) were negative except as noted. Tests for urinary urobilinogen were consistently negative.

*It would seem unlikely that this titer of 1:16,000,000 obtained on Feb. 26, 1947, is correct. The test was not confirmed.

The kidneys, adrenals and pancreas were normal. The occipital bones were both fractured in several places. The brain showed no evidence of injury or hemorrhage but marked softening and edema were present. The placenta was hypertrophic, pale and edematous.

Microscopic sections were prepared and reviewed.* The report follows:

Single sections of tissue fixed in formalin and stained with hematoxylin and eosin were available from the following tissues: lung, adrenal, spleen, placenta, diaphragm, liver, thymus and kidney. Sections of the liver and spleen were stained for iron. *Lung:* The alveoli were distended with amniotic fluid but there was no inflammatory reaction. Normoblasts and large erythroblasts were present in large numbers in the capillaries of the alveoli and also in some of the larger vessels. There were several islands of blood formation in the subpleural connective tissue. These were chiefly erythropoietic but there was also

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some leucopoiesis. *Adrenal:* Radial streaks of cells of the fetal cortex contained each a single large vacuole which looks like a fat droplet. In some places in the adult cortex the capillaries are well preserved and contain many nucleated red cells, both normoblasts and erythroblasts. *Spleen:* There was very extensive autolysis but cell nuclei could be seen throughout the section. No lymphoid follicles could be seen. There was an abnormal amount of iron within cells in circles and streaks as one sees the iron in erythroblastosis. *Placenta:* Most of the villi were small with a condensed stroma but about 20 per cent were larger with a loose, empty and possibly edematous stroma. The maternal sinusoids were empty. The capillaries of the villi were fairly well preserved and contained nucleated cells, both normoblasts and erythroblasts. The remaining organs added no information, autolysis being far advanced. From the above we can say: (1.) The fetus had attempted to breathe in utero, aspirating amniotic fluid. (2.) There had been an abnormal degree of blood destruction with deposition of iron in excess in the spleen. (3.) There was abnormal blood formation with immaturity of the red cell series in the circulating blood. From these it can be concluded that the baby almost certainly had erythroblastosis and it can be assumed that the degree of anemia was so great as to cause anoxia with resulting attempted respiration. Finally, the lesion in the adrenal is commonly seen in fetuses and newborn infants dying of erythroblastosis, particularly those with hydrops, but also sometimes occurring in other clinical forms of the disease. I do not think it possible to say for certain that the fetus suffered from hydrops.

On the basis of gross and microscopic findings a diagnosis of erythroblastosis seems justified and it is assumed that death was due to this disease. A confirming factor was the persistence of Rh antibody to a titer of 1:256 (albumin anti-D) in the mother's blood two months post partum.

The outlook for the birth of a normal child to this couple appeared hopeless. It was therefore decided to attempt to "desensitize" or "stabilize" the woman by serial, increasing injections intravenously of her husband's blood. The possibility of severe reactions was recognized as well as the probability of making this isoimmunized woman even more sensitive. The program of the procedure is presented in Table I and Fig. 1.

By the twenty-fourth injection (June 25, 1947) a maximum intravenous injection of 56 c.c. of the husband's blood was reached and it was obvious that no further increase could be undertaken without danger to the patient. Reactions proved almost as severe with the lesser amounts of 35 and 20 cubic centimeters. The patient had reached a stage of extremely marked sensitivity. In fact, she was given 5 c.c. of blood intravenously and suffered a reaction almost as severe as with 56 c.c. previously and much greater than with 43 c.c. on the way up. At this time it became apparent that the patient was pregnant, her last menstrual period having been on July 14, 1947. It was accordingly decided to give the husband's Rh-positive blood intramuscularly instead of intravenously. There were no subjective reactions to the intramuscular blood. The patient received a total of twenty-eight intravenous and forty-three intramuscular injections. From Nov. 9, 1947 (sixteenth week of pregnancy) until delivery the patient was given 3 to 5 Gm. per day orally of methionine (Meonine*).

*Meonine, brand of methionine. Kindly supplied by Wyeth & Bro., Ltd., Walkerville, Ontario, Canada.

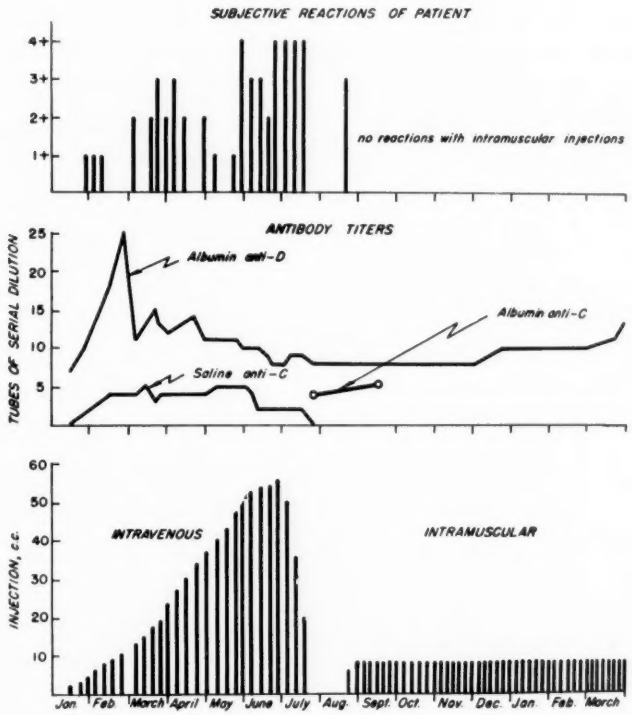

Fig. 1.—Mrs. E. N. E.; for details see Table I.

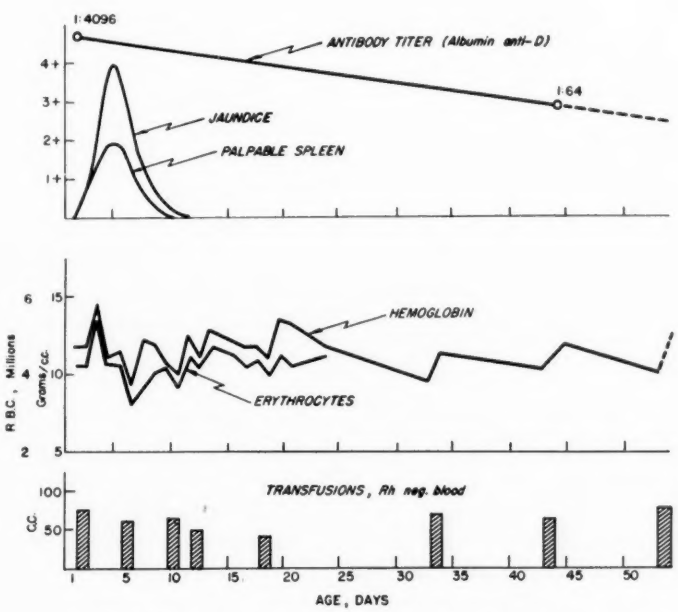

Fig. 2.—Baby L. E. E.; for details see Table II.

The expected date of delivery was April 21, 1948. The course of the pregnancy was uneventful with an average gain in weight of the mother and with fetal size, activity, and heart sounds being normal. Since the antibody titer had shown a continued rise from the twenty-third week of the pregnancy, a surgical induction with rupture of the membranes was performed on March 29,

TABLE II

DATE (1948)	WEIGHT (LB.-OZ.)	HEMOGLOBIN (GM.)	R.B.C. (MILLIONS)	W.B.C.	ALBUMIN ANTI-D	JAUNDICE	SPLEEN	TRANSFUSION
Mar. 29	5-12	11.7	4.26	15,750	1:4,096	0	0	
Mar. 30	5- 8	11.7	4.20	27,400		+	+	75 c.c.
Mar. 31	5-10	14.6	5.69			++	++	
Apr. 1	5- 9	11.1	4.26			++++	++	
Apr. 2	5- 8	11.5	4.20			+++	+	
Apr. 3	5- 6	9.3	3.26			++	+	60 c.c.
Apr. 4	5- 7	12.1				+	0	
Apr. 5	5- 8	11.0	4.09	7,450		+	0	
Apr. 6	5- 6	11.0	4.20			+	0	
Apr. 7	5- 8	10.0	3.66			+	0	65 c.c.
Apr. 8	5-10	12.5	4.45			0	0	
Apr. 9	5-11	11.0	4.20			0	0	60 c.c.
Apr. 10	5-11	12.8	4.70			0	0	
Apr. 11	5-11					0	0	
Apr. 12	5-12	12.1	4.45			0	0	
Apr. 13	5-13	11.7	4.20			0	0	
Apr. 14	5-14	11.7	4.31			0	0	
Apr. 15	5-15	11.0	3.97			0	0	40 c.c.
Apr. 16	6- 0	13.5	4.56			0	0	
Apr. 17	6- 1	13.5	4.20	8,500		0	0	
Apr. 21	6- 8	11.7	4.45			0	0	
Apr. 30	7- 4	9.5				0	0	70 c.c.
May 1		11.3				0	0	
May 10	7-13	10.2				0	0	65 c.c.
May 11		11.8			1:64	0	0	
May 21	8-14	9.1				0	0	80 c.c.
June 9		9.5				0	0	45 c.c.
June 21	10- 6	10.7				0	0	
July 7	12-14	11.5			0	0	0	
Aug. 15	14- 1	11.0						
Sept. 15	14-10	11.7						
Oct. 15	16- 8	11.3						
Nov. 15		11.7						

Data on infant (Baby L. E. E.) showing weight, blood counts, Rh antibody titer, etc. Jaundice graded from + to ++++, the latter indicating a deeply jaundiced condition. Spleen only slightly enlarged. At birth occasional erythroblasts were seen in the blood smear, none subesquently. The red blood cell fragility was normal.

Platelets, 516,600. X-rays of the skull, long bones, and chest were negative. All transfusions given were of compatible Rh-negative blood.

1948 (thirty-seventh week), with delivery of a lusty baby girl weighing 5 lb. 12 oz. (2,600 grams). The amniotic fluid was clear. The placenta was normal grossly and microscopically. On immediate thorough physical examination the infant appeared quite normal without evidence of jaundice or edema. The liver and spleen were not palpable. The following day the baby was slightly jaundiced and the tip of the spleen was palpable. The progress of the baby is recorded in Table II and Fig. 2.

The Rh tests on the baby were as follows:

(1) Cord blood at birth: "Group O. cde cde (C–, D–, E–, c+). Antihuman globulin test (Coombs[5] test) on the baby's cells was negative.

	MOTHER'S SERUM WITH BABY'S CELLS	MOTHER'S SERUM WITH PANEL	BABY'S SERUM WITH PANEL
Saline	None	None	None
Albumin	None	Anti-D—1:4,096	Anti-D—1:4,096"

This test was repeated the following day on the same sample of blood with identical results.*

(2) Two days of age: "Group O. Behaves like an Rh-negative blood, but this is so only because of very intense coating with maternal antibodies."†

(3) Three weeks of age: "Group O. Rh-negative. The cells are not now coated with maternal antibodies. Antibodies (anti-D) still demonstrable at a titer of 1:64."†

(4) Three and one half months of age: "Group O. Rh-positive. Type cDe cde (R_0 r), (C–, D+, E–, c+, C^w–)."†‡

At date of writing (March, 1949), the baby has progressed and developed quite normally.

DISCUSSION

This case is not presented with any idea of recommending the procedure carried out as a method of treatment, even though the outcome of the pregnancy was a happy one. It does, however, present several interesting features and raises some question as to the validity of the current concepts of the pathogenesis of erythroblastosis.

One interesting feature of the case is the negative antihuman globulin test for "cell coating" (Coombs[5] test) on the baby's cells at birth. Race[6] offers the following possible explanations of this: (a) The baby's cells were not adequately washed free from serum. (b) The antihuman globulin was not sufficiently active. (c) Though sensitized, the baby's cells failed to give a positive Coombs test. (d) At the time of birth the baby's cells were not sensitized and only became sensitized later.

Another feature was the consistent absence of or weak agglutination in albumin of the anti-D factor (negative prozone) in the first two to five tubes of the titrations. Levine,[7] Mohn and Witebsky[8] believe that this prozone phenomenon which usually occurs only in highly immunized individuals may be due to a third type of Rh antibody of true blocking character, in addition to saline (complete) and albumin (incomplete) antibodies. They believe that this type of blocking Rh antibody prevents agglutination by either of the others. Howard and co-workers[9] suggest that blocking antibody may act as a protection for the erythrocytes of the fetus. The hypothesis could be offered that this explains the successful outcome in this case.

A remarkably high titer of albumin antibody was obtained in this case. This was to be expected with the intensive, additional immunizations. There

*Tests performed by the Vancouver Red Cross Laboratory.

†Philip Levine, M. D.

‡Confirmed by Dr. R. R. Race, The Lister Institute, London, England, and by Dr. L. K. Diamond, The Blood Grouping Laboratory, Boston, Mass.

was no correlation of the antibody titers with the reactions of the mother (Table I and Fig. 1). The recovery of an infant with such a high content and long persistence in its blood of maternal antibodies is certainly a rarity. Wiener and co-workers[2, 10] report a case of survival of an infant with massive exchange transfusions. In none of their previous cases had an infant survived if the mother had a titer as high as blocking test to 4 units and the albumin-plasma conglutination test to 50 units. Statistics of Davidsohn and Stern[4] show that where blocking antibodies were present to 1:10 or less, 41.9 per cent of infants did not survive, and where the titer was above 1:10, 71.4 per cent did not survive. They also state, in regard to a mother with serum agglutinations to 1:29 and blocking antibodies to 1:5 at the eighth week of pregnancy, that the possibility that the antibodies were of the "carry-over" variety was the only hope in the case.

Carter[11] has very recently had considerable success in lowering antibody titers and preventing erythroblastosis by giving the mother prenatal injections intramuscularly of Rh haptens prepared by alcohol and ether extraction from pooled Rh-positive cells. The hapten[12, 13] also has been used with success in the treatment of erythroblastosis in the infant. It would seem to be a possibility in the case here presented that the breakdown and dissolution of the Rh-positive red blood cells at the sites of the intramuscular injections might have released some hapten for absorption.

SUMMARY

A report is presented of an isoimmunized Rh-negative woman. Following transfusion of her husband's Rh-positive blood and delivery of a stillborn, hydroptic infant, additional intensive immunization was brought about by intravenous injections of the husband's blood over a period of six months. An extremely high antibody titer was produced early in the course of the injections which fell rapidly and appeared to be stabilized. Pregnancy supervening and subjective reactions preventing any further intravenous injections, the patient was given a series of intramuscular injections of her husband's blood throughout the pregnancy. She was delivered of a mildly erythroblastotic infant which survived without any great difficulty. Tables and illustrations presenting the course of the mother and infant are included. Various interesting features of the case are discussed.

We would like to thank especially the Vancouver Red Cross Laboratory which performed most of our tests, Dr. R. R. Race of The Lister Institute, London, England, who confirmed the family genotypes, Dr. R. Galpin, Pediatrician, Victoria, British Columbia, who cared for the baby girl, Dr. Bruce Chown, Professor of Pediatrics, University of Manitoba Medical School, who assisted in the preparation of this report, and Dr. Philip Levine, The Rh Blood Testing Laboratory, Ortho Research Foundation, Raritan, N. J., who performed a number of the tests for us as noted in the text.

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A RE-EVALUATION OF PAPAVERINE IN THE TREATMENT OF ANGINA PECTORIS

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FOR several years papaverine has been used, both intravenously and orally, in the treatment of the anginal syndrome with conflicting reports as to its value. It is an isoquinoline alkaloid derived from opium which does not produce addiction.[1] Experimentally it has been shown in studies from this laboratory and elsewhere to be a powerful vasodilator.[2, 3, 4] It diminishes or abolishes premature systoles of ventricular origin.[5, 6] Pal[7] and Macht[8] recorded some beneficial effects with intravenous papaverine. Using oral doses of 26 mg. (3/8 gr.) daily, Evans and Hoyle[13] reported questionable benefit. In 1942 this department administered oral papaverine in doses higher than those previously used: with 400 mg. (6 gr.) daily there was apparent improvement in 75 per cent of the cases studied.[5] Since this report other authors have obtained divergent results. Swanson[9] reported improvement in eleven of twelve patients. Gray and co-workers[10] found no improvement in ten patients using daily doses of 400 mg. (6 gr.) and 800 mg. (12 gr.). White[11] and Gold[12] have both stated that the drug is of little or no value. Our clinical experience with the drug in the years since the initial optimistic report has been disappointing. This experience, plus the variable results of other investigators, demanded a critical re-evaluation of the usefulness of papaverine in the treatment of the anginal syndrome.

METHOD

The present study was carried out on patients attending the out-patient Cardiac Clinic of the Michael Reese Hospital. Each of these patients had been attending the clinic for at least one year, and all had been thoroughly investigated. The criteria for selection of patients were: (1) each individual must unquestionably suffer from angina pectoris, (2) the attacks must occur at least twice weekly and preferably more often, and (3) the patient must be sufficiently intelligent and cooperative for an evaluation to be made. In selecting cases for this study more than one hundred patients with the clinical diagnosis of angina pectoris were interviewed. Of these only twenty-seven seemed to fit our criteria as stated. Of these twenty-seven patients, fourteen were dropped as the study progressed. Of these, four improved so much during the control and placebo periods that they no longer sustained the required number of weekly attacks. The remaining ten were dropped for various reasons: consistent failure to keep appointments, inability to attend the clinic frequently because of long distance to travel, and doubt as to the accuracy of the diagnosis. The final group consisted of thirteen patients, six women and seven men, ranging in age from 35 to 73 years with an average of 64. The duration of the anginal syndrome was one to thirteen years with an average of four and one-half years. Ten patients had arteriosclerotic heart disease; two had arteriosclerotic and hypertensive heart disease; and one had hypertensive heart disease. Three had definite histories of myocardial infarction.

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A complete history and physical examination were done in each case. Laboratory data included a complete blood count, urinalysis, blood urea nitrogen, chest x-ray, fluoroscopy of the heart and lungs, and electrocardiograms taken before, during, and following papaverine therapy. The patients were seen biweekly, occasionally at three-week intervals, usually by the same physician, in an effort to establish and keep the patient-physician relationship as a constant environmental factor. Each patient was instructed to keep a diary on forms which were supplied showing the number and duration of attacks and the number of nitroglycerine tablets used each day. On each clinic visit the patient was questioned regarding subjective improvement, degree of activity indulged in during attacks, average number of blocks he could walk before anginal pain occurred, and changes in living habits and the environmental and emotional situation which might be contributory. All received nitroglycerine as required. In some instances other medication, such as phenobarbital, was used. The amounts of other medication were maintained constant. Three patients were on a maintenance dose of digitalis throughout the study.

At the beginning of the study each patient was followed for at least six weeks before either placebo or papaverine medication was administered. This period served as one of adjustment for the patient and physician. When it appeared that the patient's angina had reached a basic level, he was given placebo (lactose) capsules which appeared in all respects identical with the papaverine capsules.* These were taken four times daily before meals and at bedtime, and the patient was told that the capsules were to reduce or prevent chest pain. After not less than four weeks on placebo therapy, during which time any improvement was evaluated, capsules containing 100 mg. (1½ gr.) of papaverine hydrochloride were substituted for the placebo without the patient's knowledge. This dosage level was maintained for four weeks or more. In order to minimize the effects of spontaneous variations in the course of the disease and the psychic influence of drug administration, the patient was kept at various dosage levels of placebo or papaverine until his response appeared to have stabilized. When a definite decision could be made regarding response to the 400 mg. (6 gr.) daily dosage, the drug was increased to 600 mg. (9 gr.) a day, later to 800 mg. (12 gr.) daily. If there was any doubt about the response to papaverine, placebo capsules were substituted at the same dosage level. Finally, following cessation of placebo and papaverine therapy, each patient was followed for a further control period of at least four weeks. The total period of observation covered eight to twelve months.

CRITERIA FOR EVALUATION

The evaluation of therapy for chronic coronary insufficiency when manifested by the anginal syndrome is always a difficult task. There are at least three major reasons for this: (1) spontaneous variations in the course of the disease; (2) the noncontrollable psychic influences; (3) the absence of a reliable quantitative test to judge the effect of treatment. We believe that these factors have been mainly responsible for the variable results obtained by the different investigators who have attempted to evaluate the efficacy of papaverine in angina pectoris. The present study was undertaken with a view toward obtaining experimental conditions which would minimize the importance of the foregoing factors as much as possible. Directed toward this end were the careful selection of cooperative patients, the observation of the patient through a relatively long initial control period, and maintenance of the patient on placebo and successive dosage levels of papaverine until the response was clear.

In planning the study, consideration was given to the use of the exercise tolerance or anoxemia tests as possible objective (hence, more reliable) methods to judge the effect of treatment. These tests were not used for the following reasons:

*We are grateful to Eli Lilly & Company for supplying the papaverine and placebo capsules used in this study.

1. It would be impossible to assess the validity of changes in the patient's response to exercise or anoxemia without establishing a long control period during which these tests would have to be repeated as frequently as one or more times per week and as frequently during the placebo and drug periods. Such a program was impracticable. Furthermore, there is no evidence in the literature to indicate that either of these tests has or can be used as a fine reliable quantitative measure of the degree of coronary insufficiency.

2. Both the exercise tolerance and anoxemia tests are artificial procedures. Neither one can be considered an accurate reproduction of the total clinical state which results in angina pectoris. It was felt, therefore, that results from these tests could not be applied directly to the anginal syndrome as seen clinically. The efficacy of drug therapy in the treatment of angina pectoris can be determined only by observations on the naturally occurring syndrome.

Judging the effect of treatment in angina remains a difficult problem in clinical evaluation. When observed over periods of many months, patients with angina display wide variations in ability to walk distances and perform routine tasks, and striking differences in the number of attacks of pain suffered. Basing indications of improvement on either severity or duration of attacks or the number of nitroglycerine pills used in a given period might be misleading, although considered together these data suggest a trend. We considered the most useful criterion of the patient's course to be the number of attacks of pain suffered in a given period. It is true that with improvement the patient may increase his activity and thereby keep the attacks of pain at a constant frequency; however, the opposite is also true. Therefore, if the drug being investigated produces a striking or dramatic decrease in the patient's attacks of chest pain, there will be no doubt in the mind of the investigator as to the efficacy of his measures. Marginal results are difficult to evaluate. A dramatic result was considered to be an abrupt decrease by at least 50 per cent in the attacks of chest pain when they numbered in excess of forty per week, 75 per cent when they were between ten and forty, and 100 per cent when they were ten or less.

RESULTS

The results are summarized in Table I. All thirteen patients received a trial on 400 mg. (6 gr.) daily of papaverine. In only two patients was there a significant reduction in the number of attacks of pain. One of these (J. S.) showed a further reduction when the dosage was increased to 600 mg. (9 gr.) daily. However, at the higher level he complained of severe weakness and anorexia, and the papaverine had to be discontinued. Unfortunately the patient died suddenly before the final control period could be evaluated. The other patient (S. M.) who showed improvement on 400 mg. (6 gr.) daily was unusually sedated by the drug. Instead of his customary complaints of insomnia the patient now slept twelve to fourteen hours each night so that he no longer had frequent anginal attacks at night. This patient was so severely constipated by the papaverine that the drug had to be stopped.

Nine patients received 600 mg. (9 gr.) daily. Only the one already discussed (J. S.) showed significant decrease in the frequency of pain. Seven pa-

TABLE I

PATIENT	AGE	SEX	TYPE OF HEART DISEASE	AVERAGE WEEKLY NUMBER OF ATTACKS OF PAIN						
					PAPAVERINE PER DAY					
				CONTROL	PLACEBO	6 GR.	9 GR.	12 GR.	PLACEBO	CONTROL
E.S.	73	F	Arteriosclerotic	8 (6)*	9 (5)	7 (6)	8 (2)	8 (2)	7 (4)	7 (18)
W.M.	65	M	Arteriosclerotic	70 (11)	56 (8)	10 (4)	–	63 (6)	95 (2)	95 (12)
A.T.	57	F	Arteriosclerotic and hypertensive	4 (13)	2 (14)	2 (5)	–	–	0-1 (7)	0 (5)
N.L.	73	M	Arteriosclerotic	14 (6)	7-10 (9)	8-12 (7)	8-12 (2)	8-12 (7)	8-12 (3)	–
A.M.	57	M	Arteriosclerotic	15 (14)	15 (12)	16 (12)	23 (4)	–	21 (8)	21 (5)
M.W.	68	F	Arteriosclerotic	10 (6)	15 (5)	14 (3)	12 (7)	–	14 (2)	14 (3)
P.P.	69	M	Arteriosclerotic	72 (8)	83 (4)	95 (4)	91 (2)	81 (2)	84 (2)	90 (11)
J.S.†	67	M	Arteriosclerotic	79 (6)	64 (4)	40 (4)	25 (2)	–	–	–
O.S.‡	35	F	Hypertensive	1-2 (10)	0-1 (19)	0-1 (10)	–	–	0 (7)	0-2 (8)
R.B.§	68	F	Arteriosclerotic and hypertensive	10 (9)	8 (4)	8 (4)	13 (3)	70 (2)	55 (4)	35 (14)
A.M.	59	F	Arteriosclerotic	9 (6)	6 (6)	5 (6)	6 (2)	6 (2)	6 (8)	4 (4)
F.S.	70	M	Arteriosclerotic	17 (6)	14 (5)	17 (6)	15 (2)	18 (2)	17 (6)	18 (13)
S.M.	72	M	Arteriosclerotic	35 (12)	42 (5)	10-20 (11)	–	–	40 (10)	40 (10)

*The numbers in parenthesis indicate the duration of each observation period expressed in weeks.

†This patient died suddenly, shortly after papaverine was discontinued.

‡This patient lost 34 pounds in weight during the period of observation. The decrease in anginal attacks and improvement in ability to work paralleled weight loss.

§While taking 12 gr. of papaverine daily this patient's anginal attacks became very frequent. Papaverine was decreased to 6 gr. daily for a four-week period without change in symptoms; this was followed by a trial of placebo without significant change. During the final control period there was gradual lessening of attacks. There was no electrocardiographic evidence of myocardial infarction. This patient lost 57 pounds in weight during the period of observation, and 61 pounds in a period of one year without affecting anginal syndrome.

tients received 800 mg. (12 gr.) daily. In no case was there a clear-cut improvement in the ability to perform routine activities at any dosage level of papaverine.

Toxic symptoms were observed at the 400 mg. (6 gr.) level in two patients. In both the complaints were of drowsiness and constipation. With the dosage of 600 mg. (9 gr.) daily one patient complained of weakness and anorexia and one of headache and epigastric distress. At the 800 mg. (12 gr.) daily level one patient complained of drowsiness and two of constipation.

DISCUSSION

This series is large enough and sufficiently well controlled to warrant the deduction that large doses of papaverine do not have a uniformly beneficial effect on angina pectoris distinct from a psychic action. It is possible that the random sample selected is weighted and the negative results obtained are not typical of a larger series, but even if this were so, the present study demonstrates that papaverine administered orally is of limited value in angina pectoris. This does not preclude its trial clinically since an occasional patient may be found in whom the drug has a striking, beneficial effect without untoward side reactions.

The present study differs from our earlier study in one very important respect, that is the criterion of improvement. If this criterion of a striking or dramatic reduction in the number of attacks of chest pain were applied to the earlier study it would be found that many cases classified as definitely improved would now be considered as marginal results. Thus, using our present more critical attitude the results of the two studies would not be far apart.

Since papaverine definitely acts as a powerful coronary dilator in animals,[2] its failure to produce clinical results in man may be on the basis of one or several mechanisms. (1) Its action on the coronary vessels of man, particularly sclerotic coronaries, may be different from its action in animals. (2) Coronary dilatation in the cardiac region responsible for the anginal pain may not be great enough, or may be neutralized by dilatation of other systemic vessels with which the coronary vessels form parallel circuits. (3) Factors other than inadequate coronary flow may dominate the mechanism giving rise to anginal pain. (4) Dramatic results in patients with anginal pain, aside from psychic effects, may require a quick acting as well as a powerful coronary dilator such as nitroglycerine. Slower acting dilator drugs may permit readjustments not obtainable when a quick-acting drug is used.

CONCLUSIONS

1. The oral administration of papaverine in doses of 400 mg. (6 gr.) to 800 mg. (12 gr.) daily did not significantly reduce the frequency of attacks of angina pectoris or increase the patients' ability to perform routine activities in eleven of thirteen cases followed over a period of eight to twelve months.

2. In two patients papaverine appeared to have a beneficial effect on the anginal syndrome. In one patient the results appeared to be due to the sedative effect of the drug; in the other the cause of improvement was not certain.

3. Toxic effects were noted in two patients taking 400 mg. (6 gr.) daily, two taking 600 mg. (9 gr.) daily, and three taking 800 mg. (12 gr.) daily. Constipation and drowsiness were the most common symptoms.

4. A careful re-evaluation of the use of oral papaverine in the treatment of the anginal syndrome leads us to conclude that it is of limited value.

We are grateful to the clinic physicians for permission to use their patients in this study.

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SEMIWEEKLY TREATMENT OF SYPHILIS WITH PROCAINE PENICILLIN IN OIL

Reactions and Preliminary Results in 228 Patients

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THE superiority of penicillin over previous antisyphilitic agents has been clearly demonstrated by the results of the nationwide study of penicillin in syphilis.[1] Early penicillin preparations, administered in aqueous solution, required hospitalization of the patient for frequent injections. With the development of penicillin in peanut oil and wax by Romansky and Rittman,[2] the ambulatory treatment of syphilis was made possible, and results thus far available indicate that schedules employing daily injections of this material are at least as effective as aqueous regimens of similar duration. More recently, longer-acting repository penicillin preparations have been produced. It is the purpose of this report to describe the use of one of these, crystalline G procaine penicillin in sesame oil, administered at more prolonged intervals (semiweekly) in the ambulant treatment of syphilis.

The objectives of the present study were twofold: (1) to determine the therapeutic efficacy of procaine penicillin in oil administered semiweekly in the treatment of syphilis, and (2) to determine the practicability of a semiweekly schedule in the treatment of ambulant patients from the standpoint of case holding. The preliminary results herein presented are based on the treatment of 228 patients with all stages of syphilis. At the same time, information has been obtained on the lack of toxicity of procaine penicillin in oil, particularly in respect to the procaine fraction and its continued administration over prolonged periods.

MATERIALS AND METHODS

Procaine penicillin, a slightly soluble salt, is prepared by the chemical combination of procaine hydrochloride and sodium penicillin G.[3-5] The preparation employed (Duracillin, in oil*) is suspended in sesame oil, with dispersing agents added, and contains 300,000 units of penicillin and approximately 125 mg. of procaine per milliliter. Ninety per cent of the penicillin is penicillin G with a potency of 940 units per milligram, and at least 50 per cent of the particles are of large size, 50 μ or more in diameter.[3] Because of low solubility, large particle size, and suspension in oil, the penicillin is slowly absorbed and produces prolonged serum concentrations. Following a single intramuscular injection of 600,000 units, the dosage employed in this study, detectable serum penicillin concentrations have been observed at forty-eight hours in as many as 89 per cent of patients,[6] and excretion of penicillin in the urine continues for longer periods of time. Because of its slow absorption, procaine penicillin in oil would appear on a priori grounds to be well suited for the treatment of syphilis.

In a like manner the procaine is released slowly from its combination with penicillin and is absorbed over a period of hours. Although widely used for its local anesthetic properties, and recently administered intravenously in the treatment of arthritis and other disorders,[7] occasional instances of intolerance or sensitivity to procaine have been reported.[8]

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In the present study, because of concern in respect to the rather large amount of procaine contained in the preparation (approximately 250 mg. per 2 ml. dose), an attempt was made initially to screen patients in order to detect those whose tolerance to procaine might be low. Consequently, each patient was questioned regarding previous procaine injections, e.g., for extraction of teeth; skin tests were performed by the injection of 0.1 ml. of 2 per cent aqueous procaine solution (2 mg.) intradermally; and, in reference to systemic intolerance, 0.5 ml. (10 mg.) was injected intramuscularly. After thirty minutes of observation, an initial dose of 300,000 units of procaine penicillin in oil was injected, all subsequent injections being of 600,000 units each. On each of the first three return visits, patients were questioned by a physician regarding both local discomfort and general reactions, and the injection site was inspected and palpated.

Regimens Employed.—The results of the national study of penicillin in syphilis have shown essentially similar failure (or retreatment) rates for aqueous penicillin with schedules varying in duration from four to fifteen days, in dosage from 1.2 to 4.8 million units,* and in interval between injections from two to six hours.[9, 10] Results with penicillin in peanut oil and wax administered either daily or twice daily for eight and for fifteen days in total dosages of 4.8 to 9.6 million units have not been significantly different.[11, 12] Because prolongation rather than intensification may prove to be the important factor in the treatment of syphilis, and because of previous experience on a small scale using penicillin in peanut oil and wax, schedules involving semiweekly injections of 600,000 units of procaine penicillin in oil were chosen. Experimental support for increasing the total duration of treatment has been presented by Eagle, Magnuson, and Fleischman[13] who obtained successful results in treating rabbit syphilis with a schedule consisting of intramuscular injections of only 7 units of aqueous penicillin per kilogram of body weight over a prolonged period. It is significant that with this regimen Eagle and co-workers were at no time able to demonstrate detectable serum penicillin concentrations. Low concentrations acting over a long period of time were more effective than high concentrations acting over a short period of time. Subsequently, the same authors[14] found penicillin in peanut oil and wax to be two to fourteen times more effective than aqueous solutions in the treatment of experimental syphilis. For these reasons, therefore, the duration of treatment of patients included in the study was arbitrarily prolonged to seven and one-half weeks (total dosage, 9.0 million units), or alternately, in some patients with more serious late forms of the disease, to ten weeks (12.0 million units).

Clinical Material.—All new patients with syphilis, regardless of stage of disease, were treated with one or the other of the foregoing schedules. The diagnosis and treatment regimen in 228 patients are indicated in Table I. The division between early and late latent syphilis is based on the two-year time period.

TABLE I. DIAGNOSIS AND TREATMENT REGIMEN EMPLOYED IN 228 PATIENTS

SCHEDULE: TOTAL DOSAGE	9.0 MILLION UNITS	12.0 MILLION UNITS	
DURATION	7½ WK.	10 WK.	
DIAGNOSIS	NUMBER OF PATIENTS	NUMBER OF PATIENTS	TOTAL
Early (primary or secondary)	24		24
Early latent	22		22
Late latent	89		89
Neurosyphilis			
Early asymptomatic	1	2	3
Late asymptomatic	17	20	37
Late meningovascular	3	10	13
Paretic	1	2	3
Tabetic	8	10	18
Cardiovascular	9	3	12
Benign late (gummatous)	6		6
Congenital (interstitial keratitis)		1	1
Total	180	48	228

*And to 9.6 million units in our own material.

RESULTS

Toxicity.—

Procaine: Of forty-four patients subjected to the screening procedure for procaine toxicity, none reported previous untoward reactions to the drug. The intradermal test was interpreted as negative in forty-two and doubtful (slight erythema) in two. Although the intramuscular test dose resulted in no evidence of procaine intolerance, one patient reported suggestive symptoms following the injection of procaine penicillin in oil. This patient, a 38-year-old white woman with late neurosyphilis recently receiving treatment with the arsenicals and bismuth, was unaware of any previous procaine injection. She was one of the two patients whose skin test showed erythema and, consequently, although 10 mg. of procaine alone intramuscularly resulted in no subjective evidence of intolerance, she received only 150,000 units of procaine penicillin in oil as the initial dose. There were no immediate untoward symptoms but about ten hours later she awoke with feelings of extreme apprehension, nervousness, dyspnea, tightness in the chest, palpitation, tachycardia, and generalized numbness. These symptoms, varying in intensity but gradually diminishing, persisted for three days. Further injections of procaine penicillin in oil were refused, and treatment was subsequently carried out without difficulty with penicillin in peanut oil and wax. The symptoms described are compatible with procaine intolerance, but proof of its causal relationship is lacking.

Although the pretreatment procaine survey was discontinued after the original series of forty-four patients had been completed, all patients receiving procaine penicillin in oil were questioned on each return visit by the treatment nurse and at frequent intervals by a physician, and no further instances of suspected procaine intolerance occurred.

Penicillin: Of the 212 patients who have completed treatment, two with pre-existing dermatophytosis pedis developed, after the first injection of procaine penicillin in oil, transitory vesiculopapular eruptions limited to the feet which disappeared promptly without interruption of treatment. Five patients developed generalized pruritic eruptions (papular in two, urticarial in three) during the course of treatment. In one patient, who one year previously had developed a giant hive at the site of injection of aqueous penicillin for pneumonia, generalized urticaria appeared after the first injection of procaine penicillin in oil. In two patients the generalized eruptions appeared after the fourth injection, and in one each after the sixth and seventh injections. In two of these five patients treatment was interrupted temporarily; in three, itching was controlled with an antihistaminic drug and treatment was continued uneventfully.

These eruptions were similar to those observed previously in patients treated either with aqueous penicillin or with penicillin in peanut oil and wax. The incidence of this type of reaction following procaine penicillin in oil (approximately 2.5 per cent of patients) is less in our experience than with penicillin in peanut oil and wax (5 per cent).

The procaine penicillin in oil preparation has proved superior to penicillin in peanut oil and wax in other respects. These include ease of handling and of administration, and absence of local discomfort and of nodule formation such as was observed following the inadvertent superficial injection of penicillin in oil and wax. Rarely, and because of faulty technique of injection, transient numbness of the leg on the injected side has occurred.

In summary, in approximately 3,500 injections administered to a total of 228 patients, of whom 212* have received from fifteen to twenty injections over seven and one-half to ten week time periods, there has been only a single instance of suspected (but not proved) procaine intolerance, and an incidence of 2.5 per cent or less of generalized eruptions of the type attributed to penicillin. From the standpoint of ease of administration and absence of local discomfort, this procaine penicillin in oil preparation has proved highly satisfactory.

THERAPEUTIC EFFICACY

In a chronic infection such as syphilis, little information on the therapeutic effectiveness of an agent can be gained from a small series of patients with diverse stages of disease observed for only brief periods of time. It is to be emphasized that the data to be presented are meager, and final interpretation must wait upon future experience. Preliminary impressions of therapeutic effectiveness can be obtained (1) in early syphilis by the disappearance time of *Treponema pallidum*, by the rapidity of healing of lesions, by the serologic response, and by the occurrence of relapse; (2) by the healing of lesions in benign late syphilis; and (3) by the trend of the cerebrospinal fluid abnormalities in neurosyphilis.

Early Syphilis.—T. pallidum disappear rapidly (within twenty-four hours) from the moist lesions of early syphilis following injection of an effective antitreponemal agent. In the twenty-four patients with early syphilis treated with the semiweekly schedule (600,000 units per dose), dark-field examination was frequently impracticable at the time of the first post-treatment visit because of the advanced stage of healing. For this reason, ten patients with dark-field positive lesions of early syphilis† were observed eighteen to twenty-four hours after an initial injection of 300,000 units of procaine penicillin in oil. In each instance, *T. pallidum* were no longer present in serum expressed from previously dark-field positive lesions.‡ This corresponds with previous findings following penicillin, either aqueous or in oil and wax.

As indicated, the lesions of early syphilis promptly resolved; this is in conformance with observations following the administration of other penicillin preparations.

In respect to serologic response, the data are too meager for statistical presentation. However, as of this writing, eight of twenty-four patients have

*Sixteen patients lapsed before completing treatment.

†Not otherwise included in this report.

‡The author wishes to thank Dr. Leland Hanchett, Medical Director, Midwestern Medical Center, for these observations.

achieved seronegativity; the remainder have doubtful or low-titered quantitative tests. None have relapsed either clinically or serologically; there have been no reinfections.

It should be emphasized that none of the above-mentioned criteria, other than relapse, are reliable indicators of therapeutic efficacy. The adequacy of any therapeutic agent (in early syphilis) can be determined only by the careful and prolonged observation of large numbers of patients for evidence of clinical and serologic relapse. However, there is reason to believe that procaine penicillin in oil will prove to be at least as effective as penicillin in oil and wax.

The lesions of benign late syphilis heal after even relatively minute amounts of aqueous penicillin.[17] The prompt response of the six patients with gummas (two cutaneous, one mucosal, two visceral) treated with procaine penicillin in oil would be anticipated.

Neurosyphilis.—It has been emphasized by Dattner[18] that the most reliable indicator of therapeutic effectiveness in neurosyphilis is the response of the cerebrospinal fluid abnormalities, particularly in terms of cell count and total protein. Improvement in colloidal and quantitative complement fixation tests follows slowly and may require several years to become manifest. Of the seventy-four patients with neurosyphilis included in the present study, twenty-five have thus far had examinations of the cerebrospinal fluid approximately three to four months following completion of treatment. Evidence of "activity" of the syphilitic process before treatment is documented by the following data. Using the method devised by Reynolds,[19] but employing median values rather than means as a more representative centering constant for small series, the results of the pretreatment cerebrospinal fluid examination in these twenty-five patients were as follows: cell count = 92/3,* total protein = 64 mg. per cent, colloidal gold = 15,† complement fixation test = 16.‡ The initial post-treatment results in these same patients obtained three to four months following completion of treatment are in striking contrast: cells = 10/3, total protein = 46 mg. per cent, colloidal gold = 3, complement fixation test = 4.

The three following brief case reports are typical of the cerebrospinal fluid response in patients observed for more prolonged periods of time. Although none thus far have required re-treatment, much longer observation will of course be necessary to determine the ultimate outcome.

CASE 1.—A. B. (A-4271), a 41-year-old white man with untreated late meningovascular neurosyphilis, received 12 million units of procaine penicillin in oil (600,000 units twice weekly) between January 30 and April 6, 1948. The cerebrospinal fluid results are indicated in Table II. The blood serologic titer over the same period decreased from 600 to 120 Kahn units.

*Fuchs-Rosenthal counting chamber, 92 cells per 3.2 mm.^{3} of spinal fluid.

†Arithmetic sum of the first three tubes, e.g., 5554321000 = 15.

‡In this laboratory, the spinal fluid complement fixation test is quantitated by using decreasing amounts of spinal fluid as follows: 1.0, 0.5, 0.25, 0.12, 0.06, 0.03, 0.01, and the results of each reported. In accordance with Reynolds, reciprocal values were assigned to the various readings: 0.01=100, 0.03=33, 0.06=16, 0.12=8, 0.25=4, 0.5=2, 1.0=1.

TABLE II. CEREBROSPINAL FLUID RESPONSE IN CASE 1

DAYS BEFORE OR AFTER END OF TREATMENT	CELLS/MM.^{3}	TOTAL PROTEIN (MG. %)	COLLOIDAL GOLD	COMPLEMENT FIXATION TEST AMOUNT OF SPINAL FLUID (ML.)						
				0.01	0.03	0.06	0.12	0.25	0.5	1.0
– 66*	186/3	114	5554322100	0	0	4	4	4	4	4
+ 98	16/3	116	2211100000	0	0	±	3	3	3	4
+192	28/3	No data	0000000000	0	0	±	1	3	3	4
+290	7/3	91	1111000000	0	0	±	3	4	4	4

*In this and in subsequent tables this number refers to days before the *end* of treatment, i.e. immediately preceding the beginning of treatment.

CASE 2.—J. M. (A-7483), a 49-year-old colored man with essentially untreated (two bismuth injections seven years previously) late asymptomatic neurosyphilis of twenty years' duration, received fifteen injections of procaine penicillin in oil, total dosage 9 million units, between Dec. 19, 1947, and Feb. 6, 1948. The cerebrospinal fluid response is recorded in Table III. The blood serologic titer remained unchanged during this interval.

TABLE III. CEREBROSPINAL FLUID RESPONSE IN CASE 2

DAYS BEFORE OR AFTER END OF TREATMENT	CELLS/MM.^{3}	TOTAL PROTEIN (MG. %)	COLLOIDAL GOLD	COMPLEMENT FIXATION TEST AMOUNT OF SPINAL FLUID (ML.)						
				0.01	0.03	0.06	0.12	0.25	1.5	1.0
–107*	847/3	163	5554332000	0	3	3	3	3	4	4
+ 71	38/3	65	5553210000	No data						4
+288	5/3	48	1111000000	0	0	3	4	4	4	4

*See footnote to Table II.

CASE 3.—P. M. (A-7845) was a 37-year-old colored man with late asymptomatic neurosyphilis of twenty-one years' duration. Previous treatment administered elsewhere included fever therapy, 1 injection of Mapharsen, twelve of bismuth, and nineteen of Aldarsone between March and December of 1946. As recorded, the cerebrospinal fluid on admission here one year later showed evidence of marked activity. Between Dec. 19, 1947, and March 2, 1948, he received 12 million units of procaine penicillin in oil (600,000 units twice weekly). The cerebrospinal fluid results are recorded in Table IV. The blood serologic titer, low at the onset, remained unchanged during this observation period.

TABLE IV. CEREBROSPINAL FLUID RESPONSE IN CASE 3

DAYS BEFORE OR AFTER END OF TREATMENT	CELLS/MM.^{3}	TOTAL PROTEIN (MG. %)	COLLOIDAL GOLD	COMPLEMENT FIXATION TEST AMOUNT OF SPINAL FLUID (ML.)						
				0.01	0.03	0.06	0.12	0.25	1.5	1.0
– 87*	43/3	95	5554321000	0	4	4	4	4	4	4
+108	8/3	27	0000000000	0	0	0	2	4	4	4
+276	3/3	31	1111100000	0	0	0	4	4	4	4

*See footnote to Table II.

The results obtained thus far in patients with neurosyphilis are comparable to those previously obtained by us using aqueous penicillin in similar amounts administered in divided doses every three to four hours to hospitalized patients over two- to three-week time periods. The advantage of the present method is its applicability to ambulant patients.

It should be emphasized that the particular schedules employed in this study are empirical and that no special virtue can be claimed for semiweekly injections over the two time periods arbitrarily chosen. It seems probable that a variety of regimens, e.g., daily, thrice weekly, etc., over varying periods of time will prove equally effective.

HERXHEIMER REACTIONS

An evaluation of the Jarisch-Herxheimer phenomenon requires hospitalization of the patient, particularly in respect to the occurrence of fever. Since one of the objectives of the present study was to determine the practicability of procaine penicillin in oil in the treatment of ambulatory patients, only six patients were in the hospital at the time of their initial treatment. Three of these developed febrile reactions (tabetic neurosyphilis, 38.1° C.; paretic neurosyphilis, 39° C.; gumma of the liver, 40° C.); three did not (primary syphilis, early latent syphilis, syphilitic aortic regurgitation). The time interval between the injection of procaine penicillin in oil and the height of the fever (six to sixteen hours) was the same as with aqueous penicillin. It can be said, therefore, that Herxheimer reactions of the febrile type occur with this penicillin preparation but data as to the incidence are not available in our material.

In view of current concern over the possible occurrence of the Jarisch-Herxheimer phenomenon in cardiovascular syphilis,[15, 16] it is of interest that no clinical evidence of such a reaction was detected in any of twelve patients with either syphilitic aortic regurgitation or aneurysm, or both. Nine of these had received prior treatment (recent or remote) with the metal antisyphilitic agents; three were untreated previously. No special precautions were employed in this group of patients. In the seventy-four patients with neurosyphilis, of whom the majority had active disease as reflected by the cerebrospinal fluid abnormalities, headache after the initial injection was rare and neither severe nor prolonged.

From the standpoint of Herxheimer reactions it would appear that the majority of patients with late syphilis can be treated safely on an ambulant basis. This statement is predicated on the assumption that a careful and complete medical history and physical examination do not reveal evidence of such lesions as gumma of the larynx or of the brain where Herxheimer reactions of a serious nature might occur. In patients with paretic neurosyphilis, because of the very nature of the disease as well as because of the high incidence of Herxheimer reactions, hospital rather than ambulant treatment is to be preferred. Although no convincing evidence of the occurrence of this type of reaction has been presented in cardiovascular syphilis, the advisability of routinely instituting treatment with penicillin remains to be determined.

CASE HOLDING

Of primary importance from the public health standpoint is the number of patients who fail to complete treatment administered on an ambulatory basis. Therefore, in the evaluation of schedules for the outpatient treatment of syphilis, the success or failure of case holding is a paramount concern. Dexter,[20] in reporting the results of semi-intensive arsenotherapy (three injections of Mapharsen and one of bismuth per week for ten to fifteen weeks) at the Johns Hopkins Syphilis Clinic, recorded a lapse rate of 40 per cent. Others (cited by Dexter) using similar schedules have had equally poor success in

case holding. With penicillin in peanut oil and wax, on the other hand, lapses appear to have been distinctly less. For example, Hayman,[21] with schedules consisting of daily injections for six to ten days, reported that 87 per cent of 110 patients completed treatment without interruption. Similarly, in Chope and Malcolm's[22] series, 96.6 per cent of 437 patients completed a ten-day penicillin schedule, and Frost[23] reported that 98 per cent of 952 patients completed ambulatory treatment with penicillin, Mapharsen, and bismuth on two schedules, one of ten and one of twenty-four days' duration. It is clearly apparent that, in general, the shorter the schedule the fewer the patients who will fail to complete treatment. A second factor is the untoward reactions (or lack of them) resulting from the antisyphilitic agent employed. Other factors which need not be considered here include patient education, case-holding personnel, case-holding methods employed, etc.

Schedules used in the present study are longer in duration than those employing penicillin cited (seven and one-half and ten weeks as compared with six to twenty-four days) and the percentage of lapsed patients is, in general, slightly greater. Of the 228 patients, sixteen (7 per cent) are considered to have lapsed. Six of these lapsed only after they had received more than 6.0 million units of penicillin and probably had adequate treatment. Other things being equal, it would appear that ambulatory regimens employing penicillin will prove superior to arsenical-bismuth schedules from the standpoint of case holding.

SUMMARY AND CONCLUSIONS

1. Procaine penicillin in oil was administered semiweekly to 228 patients in the ambulatory treatment of syphilis.

2. Two arbitrary schedules of therapy have been used, 600,000 units being injected intramuscularly semiweekly for seven and one-half weeks, or alternatively for ten weeks.

3. In respect to toxicity, one instance of suspected (but not proved) procaine intolerance was observed. No evidence of chronic procaine toxicity developed.

4. Cutaneous reactions of the type attributed to penicillin occurred in five patients (less than 2.5 per cent).

5. On the basis of preliminary evidence this type of repository penicillin seems well suited for the ambulant treatment of syphilis. The criteria considered included disappearance time of *T. pallidum*, rapidity of healing of lesions, and serologic trend in early syphilis, and the response of the abnormalities of the cerebrospinal fluid in late neurosyphilis. Final evaluation of the schedules employed in the present study must await wider application and more prolonged observation.

6. No serious reactions of the Jarisch-Herxheimer type occurred.

7. From the standpoint of case holding, 93 per cent of 228 patients completed treatment, 7 per cent lapsed. In this respect the ambulant treatment of syphilis with penicillin appears to be more successful than with arsenical-bismuth regimens of comparable duration.

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HISTAMINE ANTAGONISTS

XIV. An Experimental and Clinical Study of N,N-Dimethyl-N′-2-Thiazolyl-N′-p-Methoxybenzyl-Ethylenediamine Hydrochloride (194-B)

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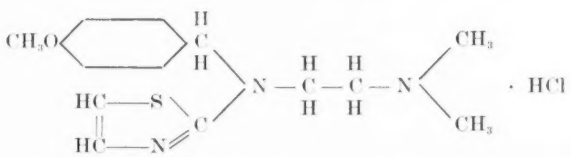
ALTHOUGH the antihistaminic drugs currently available are valuable agents for producing symptomatic relief in the management of allergic diseases, the results attained have not measured up to the earlier enthusiastic clinical claims for these substances. Their deficiencies include failure to produce relief of sufficient degree and duration, failure to benefit certain types of allergic manifestations, failure to relieve severe allergic symptoms, and the occurrence of certain toxic side reactions. For these reasons, new compounds of the ethylenediamine series continue to be synthesized for experimental assay in laboratory animals and clinical trial in human allergy.

We have been studying several of these new substances during the past year. One of them is N,N-dimethyl-N′-2-thiazolyl-N′-p-methoxybenzyl-ethylenediamine hydrochloride (194-B).* It was found to be a good drug for clinical use, combining effectiveness with a low incidence of undesirable toxic symptoms.



Laboratory studies by Kitzes, Sondern, and Neary† have shown that compound 194-B has potent antihistaminic properties and low acute and chronic toxicity. We employed several procedures to determine the antihistaminic potency of this drug in guinea pigs. Intraperitoneal injections of 0.1 mg. per kilogram protected all animals against 1MLD 100 of histamine (0.4 mg. base) given intravenously. The inhalation of a 2 per cent aerosol of this compound for five minutes at 5 pounds pressure released through a DeVilbiss nebulizer permitted the animals to remain ten minutes or longer in an aerosol of histamine obtained from a solution of 0.5 mg. per cubic centimeter, without showing dyspnea. The same animals, when unprotected, would have marked dyspnea in two minutes or less. The duration of protection conferred by the aerosol was about seventy-five minutes. The intraperitoneal injection of 5

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†Kitzes, G., Sondern, C. W., and Neary, E. R.: New Antihistaminic Compounds: N′-Arakyl Derivatives of N′Thiazolyl-N, N-Dimethylethylenediamene. I. Pharmacologic Study. (In press.)

mg. of this compound per kilogram was required to give protection against the same histamine aerosol. The duration of this action was about two and one-half hours.

In a group of ten guinea pigs which were actively sensitized with horse serum, 10 mg. per kilogram of this compound intraperitoneally were sufficient to prevent anaphylactic death from an intracardial shocking dose. In a control series of unprotected animals the mortality was 85 per cent.

We previously had devised a method to assay or quantitatively titrate the ability of an antihistaminic drug to inhibit the local reaction produced by histamine on the human skin. Essentially the method consists of applying dilutions of the drug to a series of scratches on the skin, and afterwards testing these sites with a dilution of histamine which had previously been determined to give half maximal reactions. Several antihistaminic agents can be assayed simultaneously on one subject, and twenty to forty subjects are used for each complete assay. Compound 194-B was shown by this method to be an effective inhibitor of the histamine flare and it compares favorably with several others tested.

The clinical results obtained in the symptomatic treatment of various allergic conditions with compound 194-B are tabulated (Table I). The group consisted of 249 patients of whom 148 had seasonal hay fever. The dosage in most instances was 50 mg., although a number used 100 mg. when sufficient relief was not obtained with the smaller dosage and when side effects were

TABLE I. SYMPTOMATIC RELIEF WITH 194-B

ALLERGIC DISEASE	NUMBER OF PATIENTS	SATISFACTORILY HELPED	PER CENT HELPED
Hay fever	148	94	64
Perennial rhinitis	37	15	41
Asthma	30	2	6
Cough (allergic)	12	3	25
Atopic dermatitis	9	3	33
Urticaria	9	3	33
Migraine	3	1	
Heat urticaria	1	0	
Total	249		

not a bar to increased dosage. The frequency and regularity of administration depended upon the character of the complaint. Patients with continuous symptoms, generally those with chronic perennial rhinitis, atopic dermatitis, chronic urticaria, and some with hay fever, took the medication several times daily, usually after meals. The others, whose symptoms were characterized by isolated attacks, used one dose, occasionally repeating it a few hours later if necessary. Careful and repeated observations were carried on to be reasonably certain that the symptomatic relief following medication was due to the treatment.

The best results were obtained by the hay fever patients, with 64 per cent obtaining satisfactory and consistent relief. A smaller percentage of the patients with perennial rhinitis (41 per cent) was benefited. The results

with asthma were poor and similar to what had been our experience with other antihistaminic substances. Because of the small number of the other types of allergic manifestations it is not desirable to draw conclusions until a larger number of patients is treated.

The side effects from compound 194-B were of the same nature as those produced by the older antihistaminic agents. In most instances they were mild and only rarely was it necessary to discontinue medication. Mild sedation was the most common side effect occurring in forty-one patients (16 per cent). A few patients complained of nervousness (four), gastrointestinal symptoms (four), and dermatitis (3).

SUMMARY

The laboratory experience and clinical results produced by another recently synthesized antihistaminic agent, N,N-dimethyl-N′-2-thiazolyl-N′-p-methoxybenzyl-ethylenediamine hydrochloride (194-B), are reported. This compound was found to have potent antihistaminic and antianaphylactic properties as determined by various experimental methods. Its clinical use was attended with favorable symptomatic relief in seasonal hay fever, but negligible improvement in asthma. Toxic side reactions were few in incidence and mild in degree.

THE APPRAISAL OF ANTICHOLINERGIC ACTIVITY BY PREVENTION OF METHACHOLINE-INDUCED FATAL BRONCHOSPASM IN GUINEA PIGS

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PHARMACOLOGICALLY, four etiological factors are possible in bronchial asthma: (A) A local or systemic increase of histamine concentration as in allergy, (B) hyperactivity of the parasympathetic or (C) hypoactivity of the sympathetic nervous system, and (D) toxic agents acting directly on the bronchial muscle, notably the irritants. Accordingly, antihistaminic, anticholinergic, sympathomimetic, and spasmolytic substances have been employed successfully for the relief of asthmatic attacks. Due to their common effect of relaxing the bronchial muscles, all these agents with different pharmacologic actions will alleviate spasm somewhat, regardless of the cause or causes of asthma in question. Therefore it is unreasonable in rational therapy to praise one drug or one type of drug as superior to others in the treatment of bronchial asthma in general. One agent will be more efficacious than others for a particular asthmatic condition for it will specifically antagonize the causative agent or modify a disturbed physiologic function before muscular spasm occurs.

For these reasons we have in the past two years been testing compounds both for their antihistaminic and anticholinergic properties against bronchial spasm in guinea pigs. This will assist us more precisely to elucidate the pharmacodynamics of bronchial dilating drugs in the management of asthmatic conditions. In this paper there will be presented a testing procedure and the anticholinergic activities of a number of substances as determined by this method.

EXPERIMENTAL

The procedure of Loew, Kaiser, and Moore, based on protection against the lethal effect of histamine aerosol in guinea pigs, was adapted in our work with methacholine. Acetylcholine, which has been employed with or without prostigmine by previous investigators,[1, 2] was found not very satisfactory as a cholinergic stimulant for the bronchial muscles. Acetylcholine in solution is not very stable; furthermore a high concentration of it is required to produce fatal bronchospasm in guinea pigs. Among the stimulants tested, acetyl-beta-methylcholine chloride (methacholine) and carbamyl choline chloride were found to kill guinea pigs at very low concentrations in the mist. Owing to its pure muscarinic action and close similarity in other pharmacologic properties to acetylcholine, methacholine was used in our work.[3]

The capacity of the enclosed compartment for the animals is 16 liters; half a cubic centimeter of methacholine solution (1 to 200 dilution) is sprayed

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into it in fifteen to twenty seconds. A total of ten minutes is allowed for the animals to breath the methacholine vapor. The anticholinergic agent is injected intraperitoneally fifteen minutes before the test, or, when given orally, thirty minutes beforehand. For each experiment, a control is run on animals of the same stock without medication.

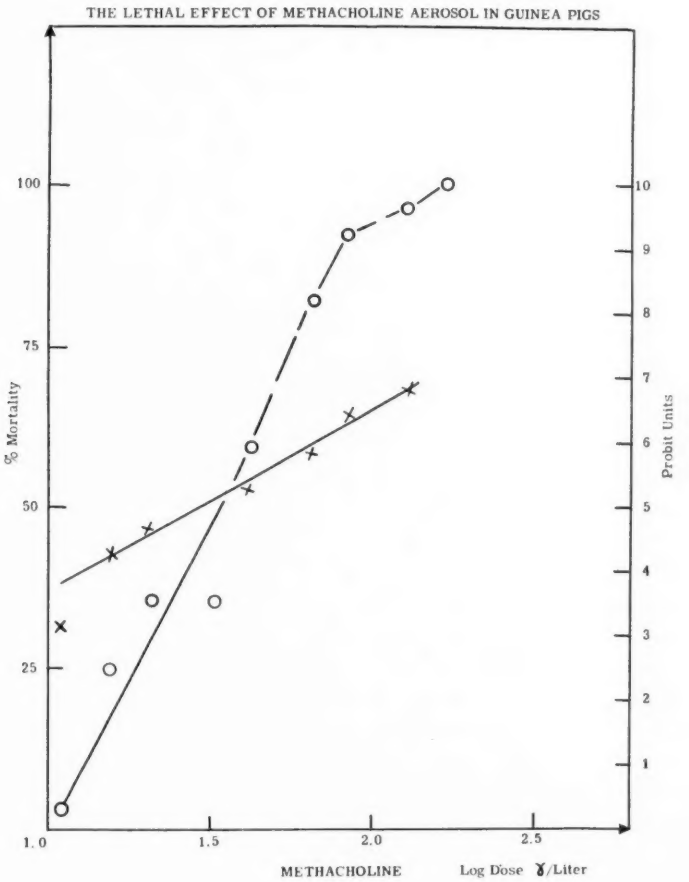

Fig. 1.

RESULTS AND DISCUSSION

In Fig. 1 are given the dose-mortality curves with methacholine aerosol in percentages and in probit units. Each point was obtained with twenty-eight animals, four in a group. One hundred fifty-eight micrograms of methacholine per liter of air (0.5 c.c. of 1 to 200 solution), which would kill around 98 per cent of animals, was chosen as a challenging dose for determining the anticholinergic effect of a compound. In Table I are recorded the anticholinergic activities of various compounds, some of which possess also antihistaminic or/and spasmolytic properties. The antihistaminic activities of these compounds are given

in the table for comparative studies. The data indicate that atropine is the most effective while aminophylline is the least effective agent for alleviating methacholine-induced bronchospasm in guinea pigs. The results are in good agreement with observations in man from vital capacity determinations.[5]

TABLE I. ACTIVITIES OF COMPOUNDS IN PREVENTING FATAL BRONCHOSPASM IN GUINEA PIGS RECEIVING METHACHOLINE OR HISTAMINE AEROSOL

COMPOUND	ANTIMETHACHOLINE PD_{50} ± S.E.* (MG./KG.)	ANTIHISTAMINE PD_{50} ± S.E.* (MG./KG.)
Atropine sulfate	0.059 ± 0.005	15
Dibutoline sulfate	0.6 ± 0.25	100 (ineffective and toxic)
N-(β-dimethylaminoisopropyl) phenothiazine HCl†	6 ± 1.6	0.3 ± 0.06
2-n-propxy-4, 6-diamino-s-triazine†	15 ± 2.0	15.5 ± 3.5
Diphenhydramine (Benadryl)	25 ± 5	2.1 ± 0.44
Papaverine HCl	29.5 ± 5.7	25
Isonipecaine	64.0 ± 15	7.3 ± 2
Khellin†	75	40
Aminophylline	100	50

*Intraperitoneal dose; average standard error calculated by the method of Miller and Tainter.[4]

†Kindly made available to us by Dr. E. Jones and Dr. C. K. Banks, Chemical Division, Research Laboratories, Parke, Davis & Company.

It is of interest to note the recent publication of Hambourger and associates on the protective action of combinations of diphenhydramine and aminophylline in guinea pigs receiving methacholine aerosol.[6] Their determinations for the individual compounds agree with ours in that large doses of the two are required to obtain a 50 per cent protection of animals inhaling a lethal concentration of methacholine aerosol. Evidently their measurements represent principally the anticholinergic effects of the two substances individually and in combinations. As judged from the antihistaminic activities and toxicities of diphenhydramine and aminophylline and their PD_{50} antimethacholine doses[6] (Table I), it is improbable that clinically the two substances relieve bronchial asthma chiefly by virtue of their anticholinergic effect. In patients whose asthmatic condition simulates that following methacholine injection,[5, 7, 8] either atropine or epinephrine should be the therapeutic agent of choice to bring about a quick relief of symptoms.

SUMMARY

A procedure is described for evaluating the anticholinergic effect of a compound in fatal bronchospasm of guinea pigs induced with methacholine aerosol.

Of the compounds studied, the decreasing order of potency was as follows: atropine sulfate, Dibutoline sulfate, n-(β-dimethylaminoisopropyl) phenothiazine HCl, 2-n-propoxy-4,6-diamino-s-triazine, diphenhydramine (Benadryl), papaverine, isonipecaine, khellin, and aminophylline.

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FURTHER STUDIES ON ENHANCEMENT OF HETEROPHILE AGGLUTINATION TITERS BY MEANS OF SERUM DILUENT

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RECENTLY we reported that heterophile and various bacterial agglutination titers were significantly enhanced by the use of serum diluent instead of saline.[1] This paper is a further report on the enhancement of heterophile agglutination titers by use of serum and other diluents in place of physiologic saline. Routine heterophile agglutination tests were done, as previously, comparing saline and pooled human serum diluents. Titrations using human ascitic fluid,* horse serum,* bovine serum,* 20 per cent bovine albumin, 20 per cent human albumin, and 10 per cent gamma globulin diluents were also carried out.

METHODS

The technique of Paul and Bunnell[2] was used in heterophile agglutination tests. The patient's serum was first inactivated by heating at 56° C. for one half hour. Serial two-fold dilutions were made of the patient's serum in saline diluent, and equal parts of 1 per cent sheep cells in saline were added to each tube. The tubes were shaken and incubated at 37° C. for two hours. Next they were removed and kept overnight in the icebox at 5° C. The titer was read as the highest dilution showing distinct visible clumps of erythrocytes.

Tests using ascitic fluid, horse serum, bovine serum, 20 per cent bovine albumin, 20 per cent human albumin, and 10 per cent human gamma globulin were carried out in an identical manner except that these various diluents were used in place of saline. Human serum, horse serum, and bovine serum diluents were inactivated by heating at 56° C. for one half hour, and all diluents were tested for the presence of heterophile antibodies prior to use. If present, heterophile agglutinins were removed by repeated absorption with sheep cells. Often, heterophile agglutinins were detected in pooled human serum and bovine serum prior to absorption with sheep cells. A diluent and saline corpuscle control tube were included in each test. The Davidsohn guinea pig kidney absorption test for infectious mononucleosis[3] was carried out in a similar manner with the patient's serum absorbed with 20 per cent guinea pig kidney suspension one hour at room temperature prior to titration.

RESULTS

Heterophile agglutination titrations comparing saline, ascitic fluid, horse serum, bovine serum, 20 per cent bovine albumin, 20 per cent human albumin, and 10 per cent human gamma globulin diluents in individuals with positive and negative clinical and hematologic† findings for infectious mononucleosis are summarized in Table I. Sheep erythrocytes suspended in saline were added to the tubes containing the various diluents. It was noted that the clumps of sheep cells in the various serum diluents were more easily dispersed on shaking than in saline.

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†We are indebted to Dr. Karl Singer of the Hematology Department of Michael Reese Hospital for hematologic studies.

TABLE I. HETEROPHILE AGGLUTINATION TITERS IN VARIOUS DILUENTS

PATIENT	STATE OF SERUM	AGGLUTINATION TITERS IN VARIOUS DILUENTS							
		SALINE	HUMAN SERUM	ASCITIC FLUID	HORSE SERUM	BOVINE SERUM	20% BOVINE ALBUMIN	20% HUMAN ALBUMIN	10% HUMAN GAMMA GLOBULIN
Positive Clinical and Hematologic Findings									
HG	Un.	1:320	1:5,120+	1:5,120	1:160	1:320	1:160	1:320	1:160
	Abs.	1:80	1:5,120	1:320	1:80	1:80	1:40	1:80	1:160
AA	Un.	1:640	1:5,120+	1:5,120	1:640	1:1,280	1:1,280	1:1,280	1:320
	Abs.	1:320	1:5,120+	1:640	1:80	1:80	1:320	1:320	1:160
RS	Un.	1:5,120	1:10,240+	1:10,240+	1:2,560	1:2,560	1:5,120	1:5,120	1:2,560
	Abs.	1:640	1:2,560	1:1,280	1:160	1:160	1:640	1:640	1:320
RW	Un.	1:160	1:640	1:640	1:160	1:160	1:320	1:80	1:40
	Abs.	1:80	1:160	1:160	1:80	1:160	1:160	1:80	1:40
BH	Un.	1:640	1:2,560	1:2,560	1:320	1:160	1:640	1:320	1:160
	Abs.	1:40	1:160	1:160	1:40	1:20	1:40	1:20	1:20
CM	Un.	1:640	1:2,560	1:5,120	1:320	1:640	1:320	–	–
	Abs.	1:160	1:640	1:1,280	1:80	1:160	1:80	–	–
SS	Un.	1:320	1:1,280	1:640	1:160	1:320	1:320	1:320	1:320
	Abs.	1:160	1:640	1:320	1:80	1:80	1:160	1:160	1:80
Negative Clinical and Hematologic Findings									
BM	Un.	1:40	1:320	1:320	1:40	1:80	1:40	1.40	1:20
	Abs.	0	0	0	0	0	0	0	0
JG	Un.	0	0	0	0	0	0	0	0
	Abs.	0	0	0	0	0	0	0	0
AL	Un.	1:40	1:160	1:80	1:20	1:20	1:40	1:40	1:20
	Abs.	0	0	0	0	0	0	0	0
BG	Un.	1:20	1:80	1:40	1:10	1:20	1:20	1:10	1:10
	Abs.	0	0	0	0	0	0	0	0
GE	Un.	1:10	1:80	1:40	1:10	–	1:10	–	–
	Abs.	0	0	0	0	–	0	–	–

Un., Unabsorbed.
Abs., Absorbed with guinea pig kidney suspension.

As shown in Table I, human serum and ascitic fluid consistently enhanced heterophile agglutination titers. On the other hand, there was no significant increase in titer using bovine serum, horse serum, 20 per cent bovine albumin, 20 per cent human albumin, and 10 per cent human gamma globulin. In fact, the titer was often one or two tubes lower than in saline. Diluents composed of 4 parts of 20 per cent human albumin and 1 part 10 per cent human gamma globulin also failed to enhance heterophile agglutination titers.

COMMENT

The results obtained in the present experiment indicate that only human serum and ascitic fluid diluents consistently enhanced heterophile agglutination titers. Horse serum, bovine serum, 20 per cent bovine albumin, 20 per cent human albumin, and 10 per cent human gamma globulin failed to enhance agglutinin titers. These results are in contrast to those obtained by others[4] relative to Rh antibody testing in which bovine albumin diluent was found to enhance Rh agglutination titers and inhibit the action of the so-called "blocking" antibody to a greater degree than serum diluent. We have confirmed these findings relative to the Rh antibody. Mixtures of human albumin and gamma globulin also failed to enhance heterophile titers. Davidsohn[5] has reported recently that bovine albumin failed to enhance heterophile agglutination titers.

SUMMARY

Heterophile agglutination titers of both unabsorbed and guinea pig kidney absorbed serum obtained from patients were significantly enhanced by the use of human serum or ascitic fluid diluent instead of saline. Horse serum, bovine serum, 20 per cent bovine albumin, 20 per cent human albumin, 10 per cent human gamma globulin, and human albumin and gamma globulin mixtures failed to enhance heterophile agglutination titers.

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LABORATORY METHODS

A COMPARISON OF EOSIN-ACETONE AND PHLOXINE-PROPYLENE GLYCOL DILUENTS IN EOSINOPHIL COUNTS

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THE potential value of direct chamber counts of circulating eosinophils as an adjunct in studies of adrenal activity has been demonstrated by Thorn.[1] Randolph[2] examined previous methods for direct eosinophil counts in 1944 and experimented with several diluents including certain ones with a propylene glycol base. He concluded that diluents consisting of eosin, acetone, and water were unsatisfactory because of lysis of leucocytes in these fluids. The present study was designed to evaluate two diluents for eosinophil counts.

The diluent used by Thorn[1] and a modification of Randolph's[2] eosinophil diluent were prepared as follows:

EOSIN-ACETONE DILUENT		PHLOXINE-PROPYLENE GLYCOL DILUENT	
Aqueous eosin, 2 per cent	5.0 c.c.	Phloxine	0.05 Gm.
Acetone	5.0 c.c.	Propylene glycol	50.0 c.c.
Distilled water	90.0 c.c.	Distilled water	50.0 c.c.

METHODS

Freshly drawn venous blood was placed in bottles containing sufficient oxalate to prevent clotting. Twenty-four white cell pipettes were filled at once with blood to the 1.0 mark. Twelve of these pipettes were filled to the 11 mark with the eosin-acetone diluent and twelve with the phloxine-propylene glycol diluent. After three, eighteen, and seventy-eight minutes respectively, four pipettes containing the eosin-acetone diluent and four containing the phloxine-propylene glycol diluent were placed in a mechanical shaker for two minutes and the eosinophils promptly counted. Thus the effect of continued contact of the blood with each diluent for five, twenty, and eighty minutes was observed. Identical studies were performed after the same oxalated blood had aged two and four hours. The oxalated blood was mixed thoroughly by gentle agitation before sampling. Both sides of a standard Spencer Bright-Line counting chamber were filled from each pipette and all the eosinophils within the ruled areas counted. Thus 18/10 cubic millimeters of blood diluted 1:10 were examined.

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Since the phloxine-propylene glycol diluent required fifteen minutes for complete staining of the eosinophils, the blood counting chambers were filled at the five-minute periods as with the eosin-acetone method, but the actual counting was delayed until fifteen minutes of staining time had elapsed.

RESULTS

The calculated eosinophil counts performed with the two diluents are listed in Table I and diagrammed in Figs. 1 and 2.

TABLE I

EOSINOPHIL COUNTS WITH EOSIN-ACETONE DILUENT

TIME AFTER DILUTION OF BLOOD	5 MIN.			20 MIN.			80 MIN.		
AGE OF BLOOD	0 HR.	2 HR.	4 HR.	0 HR.	2 HR.	4 HR.	0 HR.	2 HR.	4 HR.
Sample 1	161	161	222	66	33	44	44	5	16
Sample 2	216	127	200	88	38	11	22	5	22
Sample 3	177	138	177	50	50	38	11	11	0
Sample 4	156	172	150	44	27	16	27	11	5
Mean	178	150	187	62	37	27	26	8	11

EOSINOPHIL COUNTS WITH PHLOXINE-PROPYLENE GLYCOL DILUENT

TIME AFTER DILUTION OF BLOOD	5 MIN.			20 MIN.			80 MIN.		
AGE OF BLOOD	0 HR.	2 HR.	4 HR.	0 HR.	2 HR.	4 HR.	0 HR.	2 HR.	4 HR.
Sample 1	177	244	166	138	216	144	227	172	266
Sample 2	222	172	200	194	183	211	211	177	272
Sample 3	189	255	166	194	266	133	177	205	244
Sample 4	156	216	238	166	238	205	244	161	144
Mean	186	222	193	173	226	173	215	179	232

The eosin-acetone diluted blood showed a striking decrease in cell count with lapse of time after dilution of the blood. Using this diluent there was also a marked decrease in cell count with increasing age of the oxalated blood. The phloxine-propylene glycol method showed no significant change in count with lapse of time after dilution of the blood nor with aging of the oxalated blood.

The decrease in count with lapse of time after dilution of the blood with the eosin-acetone fluid was highly significant statistically ($p < 0.001$) as determined by analysis of variance. The analysis further revealed that the nature of this decrease was complex. The decrease in eosinophil counts obtained with the aged blood was also highly significant ($p < 0.01$). The relationship of count to age of blood was curvilinear. These results suggest that multiple factors are involved in these decreases.

Similar analysis of the values obtained with the phloxine-propylene glycol diluent revealed no significant change with time or age of blood. Hence all the phloxine-propylene glycol counts were pooled, providing a mean count of 199 eosinophils per cubic millimeter of blood with a standard deviation of 36 cells or 18 per cent. This emphasizes the large inherent error in this type of determination.

Since Randolph[2] observed the lysis of leucocytes in aqueous acetone diluents, the following additional study was undertaken in this laboratory. Blood was diluted with the eosin-acetone fluid and placed in a counting chamber with a thin cover slip and sealed with petroleum jelly. There was no change in the eosinophil count over a period of three hours. However, immediate examination of the cells under high magnification revealed the forma-

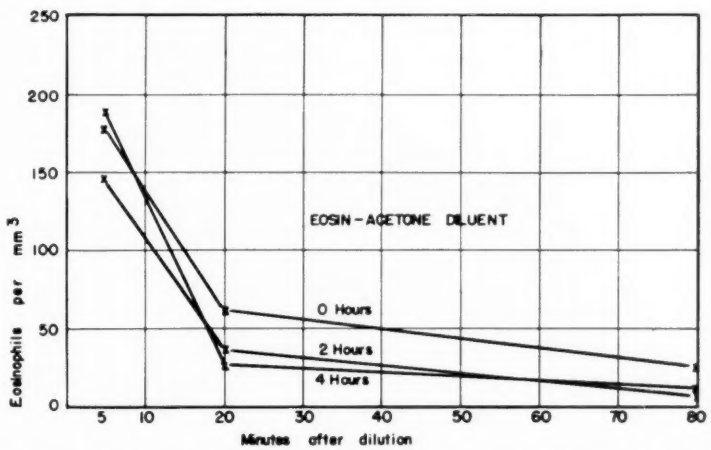

Fig. 1.—Circulating eosinophil counts performed at three time intervals, showing change in counts with passage of time and aging of oxalated blood.

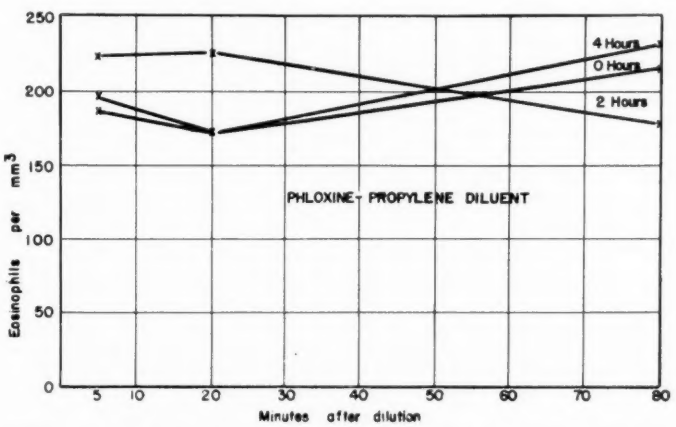

Fig. 2.—Circulating eosinophil counts performed at three time intervals after dilution, showing lack of significant change in counts with passage of time and aging of oxalated blood.

tion of blebs on the limiting membrane of the eosinophils followed by rupture of the cells. If the preparation was not agitated, the individual aggregates of eosinophilic granules could still be counted as eosinophils even though the cell membranes were disrupted. If the pipette containing blood and the eosin-acetone diluent was agitated after allowing several minutes for lysis of cells, the granules were scattered and there was an apparent decrease in eosinophil count.

The decrease in eosinophil count with passage of time and age of blood became less pronounced as the refrigerated eosin-acetone diluent aged for more than one week, suggesting that evaporation of the acetone had reduced the lytic activity of this diluent. With the phloxine-propylene glycol diluent there was no apparent lysis of eosinophils.

DISCUSSION

Using the eosin-acetone diluent, Thorn[1] recommended counting the eosinophils within three minutes of dilution of the blood. Such a procedure is suitable for a research laboratory interested in eosinophil counts. More commonly there is a delay between venipuncture and performance of the cell count which may amount to thirty or sixty minutes. Under such conditions, it is apparent that the eosin-acetone diluent is unreliable. After considerable experience with both diluents, this laboratory prefers the phloxine-propylene glycol diluent because of ease in identification of cells, decreased evaporation of diluent in the counting chamber, greater reproducibility of results, and because no refrigeration of the diluent is required.

SUMMARY

The circulating eosinophil count by the chamber method has been studied using two diluents. With an eosin-acetone mixture, there is a large and rapid decrease in cell count with passage of time after dilution of the blood and with aging of the oxalated blood. A diluent composed of phloxine in propylene glycol and water provides more consistent results and no evidence of destruction of eosinophils.

The authors are indebted to Dr. H. C. Batson and Capt. John K. Spitznagel, M.C., of the Army Medical Department Research and Graduate School for supervision and assistance in the statistical analyses.

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A SIMPLE METHOD FOR ASEPTIC GRINDING OF SMALL AMOUNTS OF TISSUE

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DURING the course of routine passages of mouse encephalitis viruses it was necessary to grind individual brains. The usual method for such grinding involves the use of a small pestle and mortar, previously wrapped in paper and sterilized. Where many pestles and mortars are required, a considerable saving of space may be effected by use of the method here described. Probably the greatest disadvantages of using pestle and mortar are the possibilities of contamination of the material being ground and the possibility of infection of the person doing the grinding.

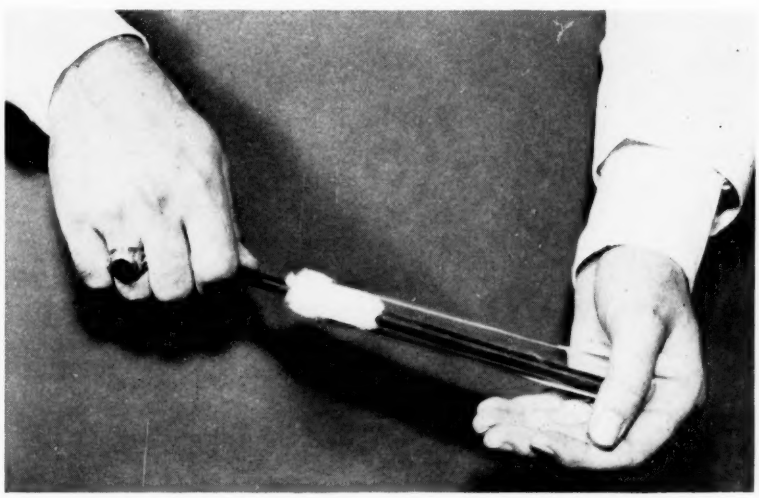

Fig. 1.—Assembly for grinding tissues.

The method employed by me calls for 6 in. Pyrex test tubes with 10 in. glass rods fitted in the tubes through the centers of the cotton stoppers. The tube-rod assemblies are sterilized in the autoclave and dried in the oven. Sterile alundum, or other suitable grinding material, may be added at the time the mouse brain is placed in the tube, or it may be added beforehand and sterilized with the assembly.

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When the brain and diluent have been added, the tube is held loosely in the palm of the left hand, with the thumb stretched across the bottom of the tube (Fig. 1). The portion of the rod showing above the cotton plug is grasped by the right hand and is made to perform a gentle whipping motion. The angle of the tube, position of the hands, etc., may be varied to the comfort of the individual employing the technique.

Obviously this technique is not limited to the grinding of brains, but may be used for lungs and other animal tissues.

PRESERVATION OF VIRUSES IN A MECHANICAL REFRIGERATOR AT –25° C.

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AS A routine, many laboratory workers who employ viruses in investigations or in diagnostic procedures store infectious material in the frozen state at about –70° C. in cabinets[1] that are refrigerated with solid carbon dioxide. Although this way of preservation is useful and effective, it has some disadvantages. Of these, the most important are: (1) the necessity for a continuous supply of solid CO_2; (2) the high annual cost of solid CO_2; and (3) the acidity caused by the diffusion of CO_2 into specimens. In many geographical areas a regular supply of solid CO_2 cannot be obtained. In areas where the refrigerant is readily available, the cost of constantly maintaining low temperatures with solid CO_2 is considerable, even when efficient cabinets are employed (the outlay may run to $100 to $200 per cubic foot of storage space per year). Although diffusion of CO_2 into specimens can be prevented by the use of sealed hard-glass containers, this procedure is cumbersome and inconvenient.

Subfreezing temperatures now can be maintained with a number of commercially available mechanical refrigerators. Certain electrically operated ones maintain temperatures in the range attained with cabinets cooled with solid CO_2, but their initial cost is high and their storage capacity not large. Other less costly refrigerators can maintain temperatures in the range of –20 to –30° C. and of these many provide large storage space.

The preservation of viruses for prolonged periods at –70° C. has been studied in a number of instances[1-3] and it is now well known that most viruses retain infectivity for long intervals when properly stored at this temperature. However, relatively little is known as to the stability of viruses at –20 to –30° C. Melnick[4] found that Y-SK and Ph poliomyelitis viruses as well as the TO Theiler virus retained their original titers when stored for twelve months at –20° C. Under the same conditions the titer of Japanese B virus decreased by one thousand fold or more.

In the present investigation, infectivity titrations were carried out with a number of different viruses which were stored in an electrically operated mechanical refrigerator for periods up to twelve months at temperatures ranging from –20 to –30° C. The results indicate that all of the viruses studied can be stored under these conditions for at least three months without causing any striking reduction in infectivity. With most of the neurotropic viruses, full infectivity was maintained for nine months. With the influenza-mumps group of viruses, infectivity was much diminished although still present after twelve

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months. It should be mentioned that during the study one refrigerator was once accidentally disconnected, and the other twice failed to operate properly. During these periods the temperature increased sufficiently so that all specimens were thawed for an interval of less than twenty-four hours.

EXPERIMENTAL

The following viruses were employed: Eastern equine encephalitis (Rockefeller Institute strain), Western equine encephalitis (Rockefeller Institute strain), Russian Far East encephalitis (No. 1 strain), Japanese B encephalitis (Nakayama strain), poliomyelitis (Lansing strain) and poliomyelitis (MEF1 strain), influenza A virus (PR8 strain), influenza B virus (Lee strain), and mumps virus (Habel strain).

The neurotropic viruses were contained in suspensions of fresh, infected mouse brains or in frozen suspensions previously stored in a CO_2 cabinet. The suspensions stored were 10^{-1} dilutions of brain in either 10 or 50 per cent normal rabbit serum which were placed either in screw-capped nitrocellulose tubes or in sealed Pyrex ampules. Influenza and mumps viruses were contained in allantoic fluid obtained from infected chick embryos. One portion of each allantoic fluid was stored in the undiluted state, the other after dilution to 10^{-1} with normal horse serum. The allantoic fluids were all kept in screw-capped nitrocellulose tubes.

In every instance a number of specimens of each virus preparation were quickly frozen by partial submersion in a mixture of ethyl alcohol and solid CO_2 before they were placed in the refrigerator at –20 to –30° C. At appropriate intervals one specimen of each frozen suspension was thawed rapidly in running tap water and an infectivity titration was performed. Titrations were carried out with serial decimal dilutions. With the neurotropic viruses the usual intracerebral technique in white mice was employed, and the LD_{50} was determined. In the case of influenza and mumps viruses the intra-allantoic technique in chick embryos was used, and the EID_{50} was determined by hemagglutination tests with allantoic fluids removed after an appropriate interval.

The refrigerators employed were commercially available models which had single stage compressor units and were capable of maintaining temperatures of –30° C. on intermittent operation. One refrigerator had a storage capacity of 20 cubic feet, the other, 16.

The results of the titrations are presented in Table I. It will be noted that all of the viruses which were suspended in 50 or more per cent normal serum retained infectivity throughout the entire period of observation. It will also be seen that the Russian Far East encephalitis virus and the MEF1 and the Lansing strains of poliomyelitis virus retained almost their original LD_{50} titers for a period of nine months. Melnick[4] also showed a similar, relative stability for other Lansing-type viruses. The Western equine encephalitis virus showed only a slight reduction in titer after nine months of storage and the titer of the Eastern equine encephalitis virus decreased only about 1.5 log units over the same period. However, the Japanese B encephalitis virus, although still infective (i.e., LD_{50} $10^{-2.6}$) after nine months, had decreased markedly in titer. This latter finding is in good agreement with the results reported by Melnick.[4]

TABLE I. EFFECT OF STORAGE AT −20 TO −30° C. ON INFECTIVITY OF VIRUSES

VIRUS	MATERIAL USED	SUSPENDED IN	ORIGINAL TITER	TITER AFTER STORAGE		
				3 MO.	6 MO.	9 MO.
			LD_{50}	LD_{50}		
Eastern equine	10^{-1} m.b.	50% n.r.s.	$10^{-9.0}$	$10^{-7.4}$	$10^{-7.2}$	$10^{-7.5}$
Western equine	10^{-1} m.b.	50% n.r.s.	$10^{-8.3}$	$10^{-8.0}$	$10^{-7.8}$	$10^{-7.8}$
Russian Far East	10^{-1} m.b. (fresh)	10% n.r.s.	$10^{-8.8}$	$10^{-5.5}$	$10^{-3.0}$	–
Russian Far East	10^{-1} m.b. (fresh)	50% n.r.s.	$10^{-9.4}$	$10^{-9.4}$	$10^{-8.8}$	$10^{-8.6}$
Japanese B	10^{-1} m.b. (fresh)	50% n.r.s.	$10^{-8.5}$	$10^{-7.5}$	$10^{-4.2}$	$10^{-2.6}$
Poliomyelitis, Lansing	10^{-1} m.b.	50% n.r.s.	$10^{-2.5}$	$10^{-2.1}$	$10^{-2.2}$	$10^{-2.2}$
Poliomyelitis, MEF1	10^{-1} m.b. (fresh)	50% n.r.s.	$10^{-3.2}$	$10^{-2.9}$	$10^{-2.3}$	$10^{-3.3}$
			EID_{50}	3 MO.	6 MO.	1 YR.
				EID_{50}		
Influenza A, PR8	10^{-1} all. fl.	90% n.h.s.	$10^{-8.8}$	$10^{-8.4}$	$10^{-5.0}$	$10^{-5.0}$
Influenza A, PR8	Undil. all. fl.	–	$10^{-8.3}$	$10^{-7.5}$	$10^{-4.7}$	$<10^{-3.0}$
Influenza B, Lee	10^{-1} all. fl.	90% n.h.s.	$10^{-7.8}$	$10^{-7.0}$	$10^{-4.5}$	$10^{-3.3}$
Influenza B, Lee	Undil. all. fl.	–	$10^{-7.8}$	$10^{-7.0}$	$10^{-3.5}$	$<10^{-2.0}$
Mumps	10^{-1} all. fl.	90% n.h.s.	$10^{-7.5}$	$10^{-7.0}$	–	$10^{-4.5}$
Mumps	Undil. all. fl.	–	$10^{-6.6}$	$10^{-5.7}$	–	$<10^{-1.0}$

m.b., Mouse brain suspension in normal rabbit serum.
n.r.s., Normal rabbit serum in saline solution.
all. fl., Allantoic fluid.
n.h.s., Normal horse serum.
EID_{50}, 50 per cent end point of virus titration in chick embryos.

It will be observed that influenza A, influenza B, and mumps viruses showed only slightly reduced EID_{50} titers after storage for three months. However, after six months of storage the titers of the influenza viruses had decreased by more than 3 log units. This was true even in the case of the suspensions which had been prepared in 90 per cent normal horse serum. After storage for one year, none of the three viruses kept in undiluted allantoic fluid showed any evidence of infectivity, and all suspended in horse serum gave titers 3 to 4 log units lower than the original titers of the suspensions.

DISCUSSION

It appears evident that a number of neurotropic viruses can be preserved by storage at −20 to −30° C. for periods of at least nine months without much loss of infectivity. Japanese B encephalitis virus seems to be the least stable of the neurotropic viruses tested; it showed a marked reduction in titer after storage for six months at this temperature. Influenza and mumps viruses exhibited a stability, under the conditions employed, which was approximately of the same order as that of Japanese B virus. Their infectivity titers also were considerably reduced following storage for six months at −25° C.

It should be emphasized that of the viruses employed, those suspended in 50 to 90 per cent normal animal serum retained infectivity sufficient to permit recovery easily after storage for at least nine months. Moreover, when such concentrations of normal serum were used none of the virus suspensions showed any striking reduction in titer after three months of storage; only those of Eastern equine and Japanese B encephalitis exhibited a decrease of 1 or more log units. It also should be stressed that the results were obtained despite the fact that the viruses had been accidentally thawed once or twice for an interval of some hours.

For many virus studies a period of storage of three months is satisfactory. In laboratories in which a CO_2 refrigerated cabinet is not available, and in areas in which a supply of solid CO_2 cannot be obtained, electrically operated, mechanical refrigerators which maintain a temperature of –20 to –30° C. may prove useful.

SUMMARY

Numerous viruses can be preserved by storage in an electrically operated mechanical refrigerator at –20 to –30° C. The infectivity titer of a number of viruses did not diminish significantly after storage for three months. In spite of the fact that some showed a marked reduction in titer after nine months, none became inactivated if stored in the presence of an adequate amount of normal serum.

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A MACERATOR FOR SMALL SAMPLES OF TISSUE

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IT IS often necessary to macerate small samples of fresh tissues for analysis before extraction with solvents. Grinding in a mortar involves transfer with an increase in the volume of washings and the risk of small losses. The Waring Blendor is an excellent instrument for the maceration of fresh tissues, but transfer to other vessels is necessary and is sometimes troublesome when the samples are small.

We have devised the highly efficient macerator, shown in Fig. 1, whose cutting blades are small enough to be inserted into a large-sized test tube. Samples of tissue are macerated in the extraction vessel, thus obviating a transfer and increasing speed and accuracy. The instrument processes organs and tissue samples of 2 to 25 grams and, if suitably modified, much larger samples could be finely macerated.

The gear housing (*A*) is made from 1/4 in. by 3/4 in. brass stock bent to form a rectangle with inside dimensions 1 1/2 in. by 2 1/4 in. Cover plates of copper sheeting are made to match the open sides. Lubricating grease covers the inside of the housing. The mounting bar (*B*) is of 3/8 in. diameter brass rod, 7 in. long, tapped into an end of the housing. Shaft (*C*) for the driving gear is coupled to an electric motor by a short length of pressure tubing. A Cenco variable speed stirrer (80 to 1,300 r.p.m.) is suitable for the purpose at the upper speed range.

The driving gear actuates gears D_1 and D_2, counterrotating the 1/8 in. diameter by 8 1/2 in. stainless steel spindles centered 3/8 in. apart. There is a step-up from the driving to the driven gears of 3 to 1, the speed of the two spindles being equal. The cutter spindles are supported by a bearing (*E*), which is 1/2 in. above the cutter blades and is mounted on a stainless steel rod support (*F*) 1/4 in. diameter and 4 1/2 in. long tapped into the bottom of the housing. The cutters, G_1 and G_2, 37 mm. by 11.5 mm., are formed from 24 gauge stainless steel in a modified "S" shape with cutting edges. A curved plate (*H*) mounted on 3/8 in. diameter rod is supported from the stand by a clamp and serves as a guide for a test tube.

The cutting blades mesh when rotated, giving fine maceration. The tendency to vortex formation by one blade is nullified by the counterrotation of the other so that fluid does not mount the sides of the vessel. The long length of the blades helps to prevent the trapping of tissue above the cutters.

The instrument is simple to operate. In this laboratory it is used to macerate rat livers for the determination of total lipids. A 28 mm. by 150 mm. Pyrex test tube containing a liver (approximately 8 to 10 grams) with sufficient acetone

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to cover the organ is placed so that the cutting blades are inserted to about 1 in. above the solvent and the test tube is held against its guide. The motor is turned on and the vessel is slowly raised against the guide until the cutters enter the solvent and end near the bottom of the tube. Maceration is complete within one

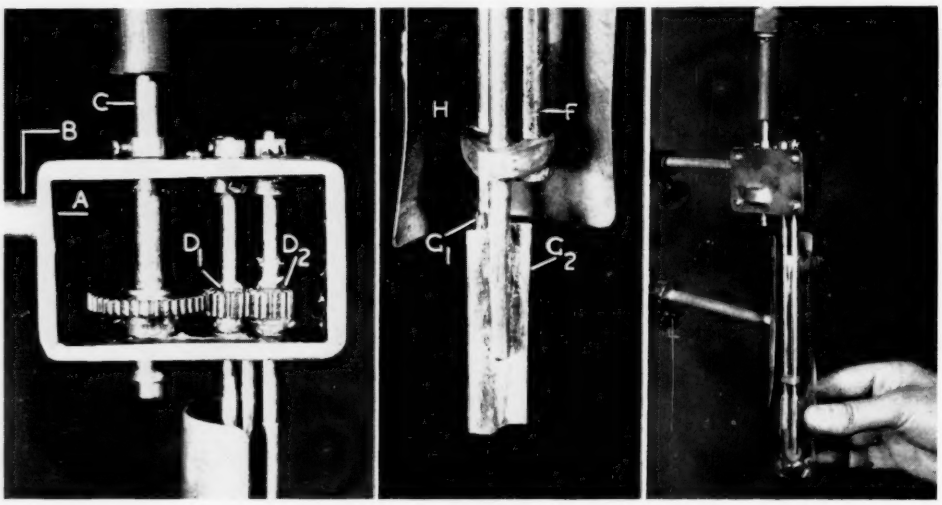

Fig. 1.

minute. The tube is partially withdrawn, the motor immediately switched off, and the cutters are quickly washed with a stream of acetone from a wash bottle. After removal of the supernatant fluid the liver may be re-extracted directly in the tube by fresh solvents.

With the instrument shown, the macerating cutters can be inserted into a vessel as small as 23 mm. in diameter, but it is obvious that the size and form of the cutters may be modified to suit many types of vessels and tissues or materials requiring maceration.

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